



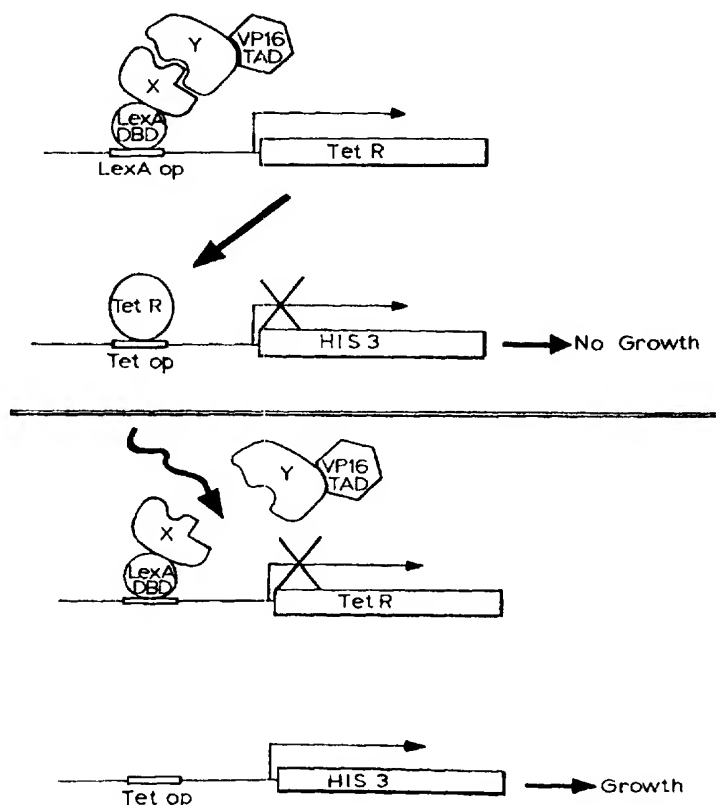
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(54) Title: METHOD TO IDENTIFY COMPOUNDS FOR DISRUPTING PROTEIN/PROTEIN INTERACTIONS

(57) Abstract

The present invention relates generally to materials and methods for identification of inhibitors of interactions between known binding partner proteins.



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METHODS TO IDENTIFY COMPOUNDS FOR DISRUPTING PROTEIN/PROTEIN INTERACTIONS

Background of the Invention

The present invention relates to a novel method to identify
5 inhibitors of protein/protein interactions.

Background

Modulation of protein/protein interactions is an attractive target
for drug discovery and development. Potential methods by which drugs can
regulate protein/protein interactions are numerous, including, for example,
10 regulation of expression of one or more of the binding proteins, modulation
of post-translational modification, and direct interference with the capacity of
one protein to bind to one or more binding partners. More importantly,
recent observations make it increasingly clear that supramolecular protein
complexes, involving two or more binding proteins, play an important and
15 essential roles in signal transduction, gene expression, cell proliferation and
duplication, and cell cycle progression. For example, in the repair of UV
damaged DNA, a so-called "repairsome" that contains over ten individual
proteins is assembled into a complex which can then carry out the necessary
repair. Likewise, gene transcription occurs through the concerted action of
20 greater than twenty proteins. Signal transduction proteins, such as receptor
protein kinases, are part of large complexes with many proteins. Contacts
through *Src* homology type 2 (SH2) domains on the receptor kinases, for
example, are noteworthy protein interaction which are part of one or more
enzymatic cascade important for many metabolic processes. Disrupting the
25 binding capacity of one or more proteins which form any of these larger
complex is therefore an important and untapped method to control action of
the overall complex.

Protein/protein interactions have been discovered and
characterized by a variety of methods: (i) standard biochemical affinity

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methods such as chromatography or co-immunoprecipitations; (ii) gel overlay methods; (iii) co-purification by traditional biochemistry; and (iv) two-hybrid analysis [Fields and Song, *Nature* **340**:245-246 (1989); Fields, *Methods: A Companion to Methods in Enzymology* **5**:116-124 (1993); U.S. Patent 5,283, 5 173 issued February 1, 1994 to Fields, *et al.*]. The most recent of these approaches, the two hybrid method, has enjoyed broad application because of its relative ease of use for gene identification from cDNA fusion libraries. [See Chien *et al.*, *Proc. Natl. Acad. Sci. (USA)* **88**:9578-9582 (1991); Dalton and Treisman, *Cell* **72**:223-232 (1993); and Durfee, *et al.*, *Genes and Devel.* 10 7:555-569 (1993)].

The two hybrid system is based on targeting and identifying a protein/protein interaction through the use of a reporter system. The described two hybrid systems either use the yeast Gal4 DNA binding domain or the *E. coli* lexA DNA binding domain and couple this region to a 15 transcriptional activator such as Gal4 or VP16 that drives a reporter like β galactosidase or HIS3.

In principle the two hybrid assay could be used for drug screening. [See WO 96/03501 and WO 96/03499.] In such a scenario, loss of β galactosidase or HIS3 activity would be identified after the yeast strain 20 is treated with a compound. In practice, however, use of the two hybrid system is technically undesirable for several reasons. In instances where the β galactosidase or HIS3 protein are employed as the reporter protein, a loss of activity is particularly difficult to detect because the expressed reporter protein is too long lived to be used in a high throughput mode. If a candidate 25 binding inhibitor compound is metabolized faster than the previously expressed reporter protein is turned over, it is difficult to detect inhibitory action of the candidate drug while a reporter protein is still active. In high throughput screening, the loss of a positive signal, for example, β galactosidase or HIS3 is impossible to detect. Present robotocized screening and detection methods 30 are simply not sufficiently sensitive or robust to detect loss of a signal.

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Thus there is a need in the art to develop a rapid screening method that gives a positive signal, as opposed to a negative signal, when a protein/protein interaction is disrupted. Such a system must be capable of using protein interactions that are initially detected by any of the above
5 mentioned approaches and must be sufficiently robust to detect a gain of function when a protein interaction is lost. In essence, the screening method must give a signal when an interaction is lost, not lose a signal when an interaction is lost. Such a system must be sensitive to subtle interactions, in particular ones that are caused by post-translational modification like protein
10 phosphorylation. Finally for large scale screening, such as high throughput screening, the system must be manipulable such that a large signal-to-noise ration can be easily detected.

Brief Summary of the Invention

In one aspect, the present invention provides materials that are
15 useful for the identification of compounds which inhibit interaction between known binding partner proteins. See Figure 1. The invention provides host cells transformed or transfected with DNA comprising: (i) a repressor gene encoding DNA binding protein that acts as a repressor protein, said repressor gene under transcriptional control of a promoter; (ii) a selectable marker gene
20 encoding a selectable marker protein; said selectable marker gene under transcriptional control of an operator; said operator regulated by interaction with said repressor protein; (iii) a first recombinant fusion protein gene encoding a first binding protein or binding fragment thereof in frame with either a DNA binding domain of a transcriptional activating protein or a
25 transactivating domain of a transcriptional activating protein; and (iv) a second recombinant fusion protein gene encoding a second binding protein or binding fragment thereof in frame with either a DNA binding domain of a transcriptional activating protein or a transactivating domain of a transcriptional activating protein, whichever domain is not encoded by the first

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fusion protein gene, said second binding protein or binding fragment thereof capable of interacting with said first binding protein or binding fragment thereof such that interaction of said second binding protein or binding fragment thereof and said first binding protein or binding fragment thereof
5 brings into proximity a DNA binding domain and a transactivating domain forming a functional transcriptional activating protein; said functional transcriptional activating protein acting on said promoter to increase expression of said repressor gene.

The invention comprehends host cells wherein the various genes
10 and regulatory sequences are encoded on a single DNA molecule as well as host cells wherein one or more of the repressor gene, the selectable marker gene, the first recombinant fusion protein gene, and the second recombinant fusion protein gene are encoded on distinct DNA expression constructs. In a preferred embodiment, the host cells are transformed or transfected with
15 DNA encoding the repressor gene, the selectable marker gene, the first recombinant fusion protein gene, and the second recombinant fusion protein gene, each encoded on a distinct expression construct. Regardless of the number of DNA expression constructs introduced, each transformed or transfected DNA expression construct further comprises a selectable marker
20 gene sequence, the expression of which is used to confirm that transfection or transformation was, in fact, accomplished. Selectable marker genes encoded on individually transformed or transfected DNA expression constructs are distinguishable from the selectable marker under transcriptional regulation of the *tet* operator in that expression of the selectable marker gene regulated by
25 the *tet* operator is central to the preferred embodiment; *i.e.*, regulated expression of the selectable marker gene by the *tet* operator provides a measurable phenotypic change in the host cell that is used to identify a binding protein inhibitor. Selectable marker genes encoded on individually transformed or transfected DNA expression constructs are provided as
30 determinants of successful transfection or transformation of the individual

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DNA expression constructs. Preferred host cells of the invention include transformed *S. cerevisiae* strains designated YI596 and YI584 which were deposited August 13, 1996 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, and assigned
5 Accession Numbers ATCC 74384 and ATCC 74385, respectively.

The host cells of the invention include any cell type capable of expressing the heterologous proteins required as described above and which are capable of being transformed or transfected with functional promoter and operator sequences which regulate expression of the heterologous proteins also
10 as described. In a preferred embodiment, the host cells are of either mammal, insect or yeast origin. Presently, the most preferred host cell is a yeast cell. The preferred yeast cells of the invention can be selected from various strains, including the *S. cerevisiae* yeast transformants described in Table 1. Alternative yeast specimens include *S.pombe*, *K.lactis*, *P.pastoris*,
15 *S.carlsbergensis* and *C.albicans*. Preferred mammalian host cells of the invention include Chinese hamster ovary (CHO), COS, HeLa, 3T3, CV1, LTK, 293T3, Rat1, PC12 or any other transfectable cell line of human or rodent origin. Preferred insect cells lines include SF9 cells.

In a preferred embodiment, the selectable marker gene is
20 regulated by an operator and encodes an enzyme in a pathway for synthesis of a nutritional requirement for said host cell such that expression of said selectable marker protein is required for growth of said host cell on media lacking said nutritional requirement. Thus, as in a preferred embodiment where a repressor protein interacts with the operator, transcription of the
25 selectable marker gene is down-regulated and the host cells are identified by an inability to grow on media lacking the nutritional requirement and an ability to grow on media containing the nutritional requirement. In a most preferred embodiment, the selectable marker gene encodes the HIS3 protein, and host cells transformed or transfected with a HIS3-encoding DNA
30 expression construct are selected following growth on media in the presence

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and absence of histidine. The invention, however, comprehends any of a number of alternative selectable marker genes regulated by an operator. Gene alternatives include, for example *URA3*, *LEU2*, *LYS2* or those encoding any of the multitude of enzymes required in various pathways for production of a nutritional requirement which can be definitively excluded from the media of growth. In addition, conventional reporter genes such as chloramphenicol acetyltransferase (CAT), firefly luciferase, β -galactosidase (β -gal), secreted alkaline phosphatase (SEAP), green fluorescent protein (GFP), human growth hormone (hGH), β -glucuronidase, neomycin, hygromycin, thymidine kinase (TK) and the like may be utilized in the invention.

In the preferred embodiment, the host cells include a repressor protein gene encoding the tetracycline resistance protein which acts on the *tet* operator to decrease expression of the selectable marker gene. The invention, however, also encompasses alternatives to the *tet* repressor and operator, for example, *E. coli trp* repressor and operator, *his* repressor and operator, and *lac* operon repressor and operator.

The DNA binding domain and transactivating domain components of the fusion protein may be derived from the same transcription factor or from different transcription factors as long as bringing the two domains into proximity permits formation of a functional transcriptional activity protein that increases expression of the repressor protein with high efficiency. A high efficiency transcriptional activating protein is defined as having both a DNA binding domain exhibiting high affinity binding for the recognized promoter sequence and a transactivating domain having high affinity binding for transcriptional machinery proteins required to express repressor gene mRNA. The DNA binding domain component of a fusion protein of the invention can be derived from any of a number of different proteins including, for example, LexA or Gal4. Similarly, the transactivating component of the invention's fusion proteins can be derived from a number of different transcriptional activating proteins, including for example, Gal4 or

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VP16. In one embodiment of the invention, polynucleotides encoding binding partner proteins CREB and CBD are inserted in plasmids pVP16-CREB and pLexA-CBD, respectively, which were deposited with the ATCC and assigned Accession Numbers ATCC 98138 and ATCC 98139, respectively.

The promoter sequence of the invention which regulates transcription of the repressor protein can be any sequence capable of driving transcription in the chosen host cell. The promoter may be a DNA sequence specifically recognized by the chosen DNA binding domain of the invention, or any other DNA sequence with which the DNA binding domain of the fusion protein is capable of high affinity interaction. In a preferred embodiment of the invention, the promoter sequence of the invention is either a HIS3 or alcohol dehydrogenase (ADH) promoter. In a presently most preferred embodiment, the ADH promoter is employed in the invention. The invention, however, encompasses numerous alternative promoters, including, for example, those derived from genes encoding HIS3, ADH, URA3, LEU2 and the like.

In another aspect, the invention provides methods to identify molecules that inhibit interaction between known binding partner proteins. In one embodiment, the invention provides a method to identify an inhibitor of binding between a first binding protein or binding fragment thereof and a second binding protein or binding fragment thereof comprising the steps of (a) growing host cells transformed or transfected as described above in the absence of a test compound and under conditions which permit expression of said first binding protein or binding fragment thereof and said second binding protein or binding fragment thereof such that said first binding protein or fragment thereof and second binding protein or binding fragment thereof interact bringing into proximity said DNA binding domain and said transactivating domain forming a functional transcriptional activating protein; the transcriptional activating protein acting on said promoter to increase

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expression of said repressor protein; said repressor protein interacting with said operator such that said selectable marker protein is not expressed; (b) confirming lack of expression of said selectable marker protein in said host cell; (c) growing said host cells in the presence of a test compound; and (d) 5 comparing expression of said selectable marker protein in the presence and absence of said test compound wherein increased expression of said selectable marker protein is indicative that the test compound is an inhibitor of binding between said first binding protein or binding fragment thereof and said second binding protein or binding fragment thereof.

10 In a most preferred embodiment, the invention provides a method to identify an inhibitor of binding between a first binding protein or binding fragment thereof and a second binding protein or binding fragment thereof comprising the steps of: (a) transforming or transfecting a host cell with a first DNA expression construct comprising a first selectable marker 15 gene encoding a first selectable marker protein and a repressor gene encoding a repressor protein, said repressor gene under transcriptional control of a promoter; (b) transforming or transfecting said host cell with a second DNA expression construct comprising a second selectable marker gene encoding a second selectable marker protein and a third selectable marker gene encoding 20 a third selectable marker protein, said third selectable marker gene under transcriptional control of an operator, said operator specifically acted upon by said repressor protein such that interaction of said repressor protein with said operator decreases expression of said third selectable marker protein; (c) transforming or transfecting said host cell with a third DNA expression 25 construct comprising a fourth selectable marker gene encoding a fourth selectable marker protein and a first fusion protein gene encoding a first binding protein or binding fragment thereof in frame with either a DNA binding domain of a transcriptional activation protein or a transactivating domain of said transcriptional activation protein; (d) transforming or 30 transfecting said host cell with a fourth DNA expression construct comprising

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a fifth selectable marker gene encoding a fifth selectable marker protein and a second fusion protein gene encoding a second binding protein or binding fragment thereof in frame with either the DNA binding domain of said transcriptional activation protein or the transactivating domain of said
5 transcriptional activation protein, whichever is not included in first fusion protein gene; (e) growing said host cell under conditions which permit expression of said first binding protein or fragment thereof and said second binding protein or fragment thereof such that said first binding protein or fragment thereof and second binding protein or binding fragment thereof
10 interact bringing into proximity said DNA binding domain and said transactivating domain reconstituting said transcriptional activating protein; said transcriptional activating protein acting on said promoter to increase expression of said repressor protein; said repressor protein interacting with said operator such that said third selectable marker protein is not expressed;
15 (f) detecting absence of expression of said selectable gene; (g) growing said host cell in the presence of a test compound of binding between said first protein or fragment thereof and said second binding protein or fragment thereof; and (h) comparing expression of said selectable marker protein in the presence and absence of said test compound wherein decreased expression of
20 said selectable marker protein is indicative of an ability of the test compound to inhibit binding between said first binding protein or binding fragment thereof and said second binding protein or binding fragment thereof such that said transcriptional activating protein is not reconstituted, expression of said repressor protein is not increased, and said operator increases expression of
25 said selectable marker protein.

The methods of the invention encompass any and all of the variations in host cells as described above. In particular, the invention encompasses a method wherein: the host cell is a yeast cell; the selectable marker gene encodes **HIS3**; transcription of the selectable marker gene is
30 regulated by the *tet* operator; the repressor protein gene encodes the

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tetracycline resistance protein; transcription of the tetracycline resistance protein is regulated by the *HIS3* promoter; the DNA binding domain is derived from LexA; and the transactivating domain is derived from VP16. In another embodiment, the invention encompasses a method wherein: the host
5 cell is a yeast cell; the selectable marker gene encodes *HIS3*; transcription of the selectable marker gene is regulated by the *tet* operator; the repressor protein gene encodes the tetracycline resistance protein; transcription of the tetracycline resistance protein is regulated by the alcohol dehydrogenase promoter; the DNA binding domain is derived from LexA; and the
10 transactivating domain is derived from VP16.

In alternative embodiments of the invention wherein the host cell is a mammalian cell, variations include the use of mammalian DNA expression constructs to encode the first and second recombinant fusion genes, the repressor gene, and the selectable marker gene, and use of selectable
15 marker genes encoding antibiotic or drug resistance markers (i.e., neomycin, hygromycin, thymidine kinase).

There are at least three different types of libraries used for the identification of small molecule modulators. These include: (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries
20 comprised of random peptides, oligonucleotides or organic molecules.

Chemical libraries consist of structural analogs of known compounds or compounds that are identified as "hits" via natural product screening. Natural product libraries are collections of microorganisms, animals plants or marine organisms which are used to create mixtures for
25 screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Combinatorial libraries are composed of large numbers of peptides, oligonucleotides or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning or
30 proprietary synthetic methods. Of particular interest are peptide and

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oligonucleotide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, polypeptide libraries.

The utility of the various aspects of the invention is manifest.

5 Host cells of the invention are useful to demonstrate *in vivo* binding capacity of both known and suspected binding partner proteins in a recombinant system. Such an expression system permits systematic analysis of the structure and function of a particular binding protein, thus permitting identification and/or synthesis of potential modulators of the physiological
10 activity of the binding proteins. The methods of the invention are particularly useful to identify and improve molecules which are capable of inhibiting specific and general protein/protein interactions. Inhibitors identified by the methods of the invention can then be examined for utility *in vivo* as therapeutic and/or prophylactic medicaments for conditions associated with
15 various protein/protein interactions.

Description of the Drawing

Figure 1 describes the mechanics of the split hybrid assays.

Detailed Description of the Invention

The present invention relates generally to methods designated
20 split hybrid assays to identify inhibitors of protein/protein interactions and is illustrated by the following examples describing various methods for making and using the invention. In particular, Example 1 relates to construction of various plasmids and expression constructs utilized in the invention. Example 2 described generation of various yeast transformants used to identify inhibitor
25 compounds. Examples 3, 4, 5 and 6 address use of the split hybrid assay to examine CREB/CBD binding, Tax/SRF binding, CKI/CREB binding and AKAP 79 binding to various partner protein, respectively. Example 7 describe general application of the split hybrid assay. Example 8 relates to

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use of the split hybrid assay for weakly interacting binding partners. Example 9 describes general assay methods. Example 10 addresses use of the split hybrids assay to identify agents that prevent receptor desensitization and drug tachyphylaxis.

5

Example 1 Plasmid Construction

In the examples that follow, various plasmid constructs were utilized as described. To simplify discussion of the exemplified assays, this example describes construction of the various plasmids used in the following examples. For clarity, the plasmids are grouped according common features relating to their applications in the assays later discussed.

I. Plasmids Encoding Reporter Gene HIS3

A. pRS303/1xtetop-MluI

One copy of the *tet* operator sequence was engineered into position -53 in the *HIS3* promoter of pRS313 [Sikorski, R.S. *et al.*, *Genetics* 122:19-27 (1989)] by using the polymerase chain reaction (PCR). Two primary PCR reactions using pRS313 as a template were performed which utilized a 5'-terminal oligonucleotide designated Eco47III-5' and a 3'-inner oligonucleotide designated Tetop internal 3' to yield a primary 5'-PCR product and a 5'-inner oligonucleotide designated Tetop internal 5' and a 3'-terminal oligonucleotide designated Nhe I 3' to yield a primary 3'-PCR product.

Eco47 III-5'

SEQ ID NO: 1

5'-TTGGTGAGCGCTAGGAGTCACTGCCAG

Tetop int. 3'

SEQ ID NO: 2

5'-TATACTCTATCAATGATAGAGTAATTCATTATGTGATAATGCC

Tetop int. 5'

SEQ ID NO: 3

5'-ATTACTCTATCATTGATAGAGTATATAAAGTAATGTGATTTC)

Nhe I 3'

SEQ ID NO: 4

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5'-AATTCTGCTAGCCTCTGCAAAGC

5' and 3' inner oligonucleotides contain complementary sequence such that 3' sequence of the primary 5' PCR product overlaps with 5' sequence of the primary 3' PCR product. The 5' terminal oligonucleotide contains the restriction site *Eco47III* while the 3' terminal oligonucleotide contains the restriction site *NheI* in order to facilitate subsequent subcloning. The primary PCR reactions were performed with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using reaction conditions described by the manufacturer. PCR products were isolated by Bio101 (Vista, CA) Gene Clean III gel extraction.

10 The primary 5' and 3' PCR products were then combined in a second PCR reaction and amplified using the 5'- and 3'- terminal oligonucleotides, *Eco47III*-5' and *NheI* 3'. The second PCR reaction was performed with *Vent* DNA polymerase (New England Biolabs, Beverly, MA) using reaction conditions described by the manufacturer, except that the reactions were supplemented with 4 mM Mg^{2+} . The final PCR product contained one *tet* operator sequence inserted into position -53 of the *HIS3* promoter and nucleotides 52-48 deleted in the construction. The final PCR product was isolated, digested with *Eco47III* and *NheI* and cloned into pRS313 previously digested with *Eco47III* and *NheI*. The resulting plasmid was designated

20 pRS313/1xtetop. DNA sequencing confirmed the presence of one copy of the *tet* operator sequence in pRS313/1xtetop and confirmed integrity of the *Eco47III* and *NheI* junctions.

A *MluI* restriction enzyme site was engineered into position -22 in the *HIS3* promoter of pRS313/1xtetop by utilizing PCR using *Vent* DNA polymerase using pRS313/1xtetop as template. One PCR construct was

25 amplified using the 5' terminal oligonucleotide *Eco47 III*-5' (SEQ ID NO: 1) containing an *Eco47III* restriction site and a 3'-oligonucleotide designated *MluI* 3' containing a *MluI* restriction site.

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Mlu I 3' SEQ ID NO: 5
5'-CGCACGCGTCGAAGAAATCACATTACTTTATATA

A second PCR product was amplified using the 3'-terminal oligonucleotide Nhe I 3' (SEQ ID NO: 4) containing a *NheI* restriction site and a 5'-oligonucleotide designated Mlu I 5' containing a *MluI* restriction site.

Mlu I 5' SEQ ID NO: 6
5'-CGCACGCGTATACTAAAAAATGAGCAGGCAAG

The first PCR product was isolated and digested with *Eco47III* and *MluI*, while the second PCR product was isolated and digested with *MluI* and *NheI*. These digested products were isolated and ligated in a triple ligation with pRS313 previously digested with *Eco47III* and *NheI*. The resulting plasmid was designated pRS313/1xtetop-MluI. DNA sequencing confirmed the presence of the *MluI* site in pRS313/1xtetop-MluI and confirmed that integrity of the *Eco47III* and *NheI* junctions were maintained.

15 A pRS303/1xtetop-MluI plasmid was constructed by first removing the *Eco47III*/*NheI* fragment containing the altered *HIS3* promoter from the pRS313/1xtetop-*MluI* vector and ligating the isolated fragment into pRS303 previously digested with *Eco47III* and *NheI*. DNA sequencing confirmed proper insertion of the *Eco47III*/*NheI* fragment.

20 B. pRS303/2xtetop-LYS2

One copy each of the *tet* operator sequence was engineered into positions -53 and -22 in the *HIS3* promoter of pRS303 [Sikorski, *et al.*, *Genetics* 122:19-27 (1989)]. PCR was utilized to engineer one copy into position -53 which resulted in plasmid pRS303/1xtetop. To insert the second copy, a *Mlu*I site was introduced at position -22 in the *HIS3* promoter using PCR. The new plasmid was designated pRS303/1xtetop-*Mlu*I.

The *tet* operator was created by annealing two complementary

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oligonucleotides tetop-1 and tetop-2.

tetop-1 SEQ ID NO: 7
5'-CGCGTACTCTATCATTGATAGAGTA;

5 tetop-2 SEQ ID NO: 8
5'-ATGAGATAGTAACTATCTCATGCGC

When annealed, the *tet* operator sequence contains flanking *Mlu*I sites. Both oligonucleotides were phosphorylated using *T4* polynucleotide kinase (Gibco BRL, Grand Island, NY) at 37°C for one hour and annealed by first heating at 70°C for 10 minutes and then cooling to room temperature. The annealed
10 oligonucleotides were isolated and ligated into pRS303/1xtetop-*Mlu*I previously digested with *Mlu*I. The resulting plasmid was designated pRS303/2xtetop. DNA sequencing confirmed insertion of one copy of the *tet* operator sequence in the *Mlu*I site.

The *LYS2* gene was digested from pLYS2 [Hollenberg, S.M.
15 *et al.*, *Mol. Cell. Biol.* 15:3813-3822 (1995)] with *Eco*RI and *Hind*III and the isolated fragment blunt ended using the large fragment of DNA polymerase I (Gibco BRL, Grand Island, NY). Phosphorylated *Sst*I linkers (New England Biolabs, Beverly, MA) were ligated to the fragment, the fragment digested with *Sst*I, and the resulting fragment ligated into pRS313 previously digested
20 with *Sst*I. The resulting plasmid was designated pRS313/LYS2.

The *LYS2* fragment was removed from pRS313/LYS2 with *Sst*I digestion and inserted into pRS303/2xtetop previously digested with *Sst*I. The resulting plasmid was designated pRS303/2xtetop-LYS2.

Similarly, the *LYS2* *Sst*I fragment was inserted into
25 pRS303/1xtetop-*Mlu*I previously digested with *Sst*I yield pRS303/1xtetop-*Mlu*I-LYS2.

C. pRS303/3xtetop-LYS2

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Two copies of the tet operator sequence were created by self-annealing a palindromic oligonucleotide Tetop 2x with itself.

Tetop 2x SEQ ID NO: 9
5'-CGCGTACTCTATCATTGATAGAGTCTAGACTCTATCAATGATAGAGTA

5 The annealed oligonucleotide contained flanking *Mlu*I sites. The oligonucleotide was phosphorylated, annealed, and isolated as above. The isolated annealed and *Mlu*I-digested oligonucleotide was ligated into pRS303/1xtetop-*Mlu*I-LYS2 previously digested with *Mlu*I to yield pRS303/3xtetop-LYS2. The presence of two copies of the *tet* operator
10 sequence in the *Mlu*I site was confirmed by DNA sequencing.

D. pRS303/4xtetop-LYS2 and pRS303/8xtetop-LYS2

Three or seven copies of the *tet* operator were created using PCR with *Vent* DNA polymerase as described above. Plasmid pUHC-13-3 [Grossen and Bujarg, *Proc. Natl. Acad. Sci. (USA)* 89:5547-5551 (1992)] was used as template DNA using 5'- and 3'- oligonucleotides, Mlu I/Sph I 5' and Mlu I Sph I 3', containing an exterior *Mlu*I restriction enzyme site nested internally by a *Sph*I restriction enzyme site.

Mlu I/Sph I 5' SEQ ID NO: 10
5'-GCGACGCGTGCATGCCGTCTTCAAGAATTCCTCGAG

20 Mlu I Sph I 3' SEQ ID NO: 11
5'-GCGACGCGTGCATGCCCACCGTACACGCCTACTCGA

The PCR products were separated on an agarose gel and the ladder of different sized DNA fragments was isolated, digested with *Mlu*I, and ligated into the *Mlu*I restriction site of pRS303/1xtetop-*Mlu*I-LYS2. DNA sequencing revealed that either three or seven copies of tet operators were inserted into the *Mlu* site of pRS303/1xtetop-*Mlu*I-LYS2 to provide either pRS303/4xtetop-LYS2 or pRS303/8xtetop-LYS2.

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E. pRS303/6xtetop-LYS2 and pRS303/10xtetop-LYS2

A *Sph*I restriction enzyme site was introduced at position -85 in the *HIS3* promoter of pRS303/3xtetop-LYS2 using PCR with *Vent* DNA polymerase as described. Plasmid pRS303/3xtetop-LYS2 was used as a template DNA. A first fragment was amplified using the 5'-terminal oligonucleotide Eco47 III-5' (SEQ ID NO: 1) described above containing an *Eco47III* restriction site and a 3'-oligonucleotide Sph I 3' containing a *Sph*I restriction site.

10 Sph I 3' SEQ ID NO: 12
5'-CATGGCATGCAAAAAAAAAAGAGTCATCCGCTAGG

A second PCR product was amplified using the 3'-terminal oligonucleotide Nhe I 3' (SEQ ID NO: 4) described above containing a *NheI* restriction site and a 5'-oligonucleotide containing a *SphI* restriction site.

Sph I 5' SEQ ID NO: 13
15 5'CATGGCATGCTTAGCGATTGGCATTATCACAT

The PCR products were isolated as described above. The first PCR product was digested with *Eco47III* and *SphI*, and the second PCR product was digested with *SphI* and *NheI*. Both digestion products were ligated in a triple ligation along with pRS303/3xtetop-LYS2 previously digested with both *Eco47III* and *NheI*. The resulting plasmid was designated pRS303/3xtetop-SphI-LYS2. The presence of the *SphI* site in pRS303/3xtetop-SphI-LYS2 was confirmed by DNA sequencing analysis.

Three copies of *tet* operators were isolated as a single fragment by digesting pRS303/4xtetop-LYS2 with *Sph*I. The isolated fragment was
25 ligated into the *Sph*I site of pRS303/3xtetop-*Sph*I-LYS2 to yield pRS303/6xtetop-LYS2. The presence of three additional copies of the *tet* operator in pRS303/6xtetop-LYS2 at the *Sph*I site was confirmed by DNA

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sequencing.

Seven copies of *tet* operators were isolated as a single fragment by digesting pRS303/8xtetop-LYS2 with *Sph*I. The isolated fragment was ligated into the *Sph*I site of pRS303/3xtetop-*Sph*I-LYS2 to yield
5 pRS303/10xtetop-LYS2. The presence of seven additional copies of the *tet* operator in pRS303/10xtetop-LYS2 at the *Sph*I site was confirmed by DNA sequencing.

F. pRS313/MluI and pRS303/MluI

A *Mlu*I restriction enzyme site was engineered into position -22
10 in the *HIS3* promoter of pRS313 utilizing PCR and *Vent* DNA polymerase as noted above. Plasmid pRS313 was used as a template for these PCR reactions. One PCR construct was amplified using the 5' terminal oligonucleotide *Eco*47III-5' (SEQ ID NO: 1) containing an *Eco*47III
restriction site and a 3' oligonucleotide *Mlu*I 3' (SEQ ID NO: 5) containing
15 a *Mlu*I restriction site. A second PCR product was amplified using the 3' terminal oligonucleotide *Nhe*I 3' (SEQ ID NO: 4) containing a *Nhe*I
restriction site and the 5' oligonucleotide *Mlu*I 5' (SEQ ID NO: 6) containing
a *Mlu*I restriction site. The first PCR product was isolated and digested with
*Eco*47III and *Mlu*I, while the second PCR product was isolated and digested
20 with *Mlu*I and *Nhe*I. The digested products were partially purified and joined in a triple ligation with pRS313 which had been previously digested with
*Eco*47III and *Nhe*I. The resulting plasmid was designated pRS313/MluI.
DNA sequencing confirmed the presence of the *Mlu*I site in pRS313/MluI and
to confirm the integrity of the *Eco*47III and *Nhe*I junctions.

25 pRS303/MluI was constructed in exactly the same manner as pRS313/MluI except that pRS303 was used in place of pRS313.

G. pRS313/1xtetop

See above wherein pRS313/1xtetop is an intermediate in the

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construction of pRS303/1xtetop-MluI.

H. pRS313/MluI-1xtetop and pRS303/MluI-1xtetop

One copy of the *tet* operator sequence was created by annealing two complementary oligonucleotides tetop-1 and tetop-2 (SEQ ID NO: 7 and
5 SEQ ID NO: 8). The annealed *tet* operator sequence contains flanking *Mlu*I sites. The oligonucleotides were phosphorylated using *T4* polynucleotide kinase (Gibco BRL, Grand Island, NY) at 37°C for one hour and annealed by first heating at 70°C for 10 minutes followed by cooling to room temperature. The annealed oligonucleotides were isolated and ligated separately into *Mlu*I-
10 digested pRS313/*Mlu*I and pRS303/*Mlu*I, the resulting plasmids being designated pRS313/*Mlu*I-1xtetop and pRS303/*Mlu*I-1xtetop. DNA sequencing confirmed the presence of one copy of the *tet* operator in the *Mlu*I sites of both plasmids.

In order to produce plasmids bearing multiple copies of the *tet*
15 operator, annealed oligonucleotides described above were ligated together overnight at 16°C. After isolation of the ligation products, they were inserted into the *Mlu*I of pRS313/*Mlu*I. DNA sequencing analysis confirmed that one clone, pRS313/*Mlu*I-4xtetop, was produced which contained four copies of *tet* operator in the *Mlu*I site. However, upon further examination of this clone
20 it was discovered that it had been subjected to a recombination event and was therefore not useful for further cloning steps. Continued attempts to insert multiple copies of the *tet* operator into the *Mlu*I site of pRS313/*Mlu*I by ligating multimers of the *tet* operator have been unsuccessful.

I. pRS313/1xtetop-MluI

25 See above wherein construction of pRS313/1xtetop-MluI was an intermediate in the construction of pRS303/1xtetop-MluI.

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J. pRS313/2xtetop

One copy of the *tet* operator sequence was created using annealed complementary oligonucleotides tetop-1 and tetop-2 (SEQ ID NO: 7 and SEQ ID NO: 8). Annealed oligonucleotides were ligated into the *MluI* site of pRS313/1xtetop-*MluI* to yield pRS313/2xtetop. DNA sequencing confirmed the presence of two copies of the *tet* operator in the *MluI* site.

K. pRS303/2xtetop

See above wherein pRS303/2xtetop was an intermediate in the construction of pRS303/2x/tetop-LYS2.

10 L. pRS313/LYS2 and pRS313/LYS2

The *LYS2* gene was digested from pLYS2 with *EcoRI* and *HindIII* digestion. The *EcoRI/HindIII* fragment was blunt ended using the large fragment of DNA polymerase I (Gibco BRL, Grand Island, NY) and ligated with phosphorylated *SstI* linkers (New England Biolabs, Beverly, MA).
15 The resulting fragment was digested with *SstI* and ligated into pRS313 previously digested with *SstI*. The resulting plasmid was designated pRS313/LYS2. Because the *LYS2* fragment was shown to have inserted into pRS313 in both orientations, plasmids with the *LYS2* gene in both orientations were transformed separately into the yeast strain SEY6210 α (*MAT α* *leu2-3,112 ura3-52 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ 9* [Robinson *et al.*, *Mol. Cell. Biol.* 8:4936-4948 (1988)]. Both clones allowed the yeast to grow in the absence of lysine indicating that orientation of the *LYS2* gene in pRS313 did not affect the expression of an active gene.

25 The *LYS2* fragment was removed from pRS313/LYS2 with *SstI* and ligated into the *SstI* site of:

pRS313/1xtetop-*MluI* giving plasmid pRS313/1xtetop-*MluI*-LYS2,
pRS313/2xtetop giving plasmid pRS313/2xtetop-LYS2,

pRS303/1xtetop-MluI giving plasmid pRS303/1xtetop-MluI-LYS2, and pRS303/2xtetop giving plasmid pRS303/2xtetop-LYS2.

II. Plasmids Encoding Reporter Gene TetR

A. pRS306/HIS3:TetR/Term

5 The 5' promoter sequence of the yeast *HIS3* gene, encompassing nucleotides -75 to +23, was ligated to the translational start of *TetR*. In addition, the DNA sequence encoding the simian virus 40 (SV40) large T antigen nuclear localization signal was ligated in frame with the nucleotide sequence encoding the last amino acid residue of TetR. The
10 chimeric fragment was created by the same PCR strategy as described above.

The *HIS3* promoter fragment, the primary 5'-PCR product, was amplified by PCR from plasmid p601 [Grueneberg, D.A., *Science* **257**:1089-1095 (1992)] using a 5'-terminal oligonucleotide T7 Promoter primer and a 3'-inner oligonucleotide 3'-TetR inner primer.

15 T7 Promoter primer SEQ ID NO: 14
5'-TAATACGACTCACTATATAGGG

3'-TetR inner primer
5'-TCTAGACTTTGCCTTCGTTTATC

The primary 3' PCR product containing the *TetR* coding sequence was amplified from pSLF104 [Forsburg, *Nucl. Acid. Res.* **21**:2955-2956 (1993)] with a 5'-inner oligonucleotide 5'-TetR inner primer and a 3'-terminal oligonucleotide 3'-TetR terminal primer.

5'-TetR inner primer SEQ ID NO: 16
5'-CGAAGGCAAAGATGTCTAGATTAGATAAAAG

25 3'-TetR terminal primer SEQ ID NO: 17
5'-CGCGGATCCGCTTTCTCTTCTTTTTGGAGACCCACTTTCACATTTAAG

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An *EcoRI* site derived from the p601 fragment and a *BamHI* site in the 3'-terminal oligonucleotide were used in subsequent subcloning. The PCR products were gel-purified and amplified in a second PCR reaction with 5'- and 3'- terminal oligonucleotides, T7 Promoter primer (SEQ ID NO: 14) and
5 3'-TetR terminal primer (SEQ ID NO: 17). The secondary PCR product was isolated, digested with *EcoRI* and *BamHI*, and ligated into pRS306/Term previously digested with *EcoRI* and *BamHI*. The resulting plasmid was designated pRS306/HIS3:TetR/Term which comprises the complete TetR coding sequence in frame with sequences encoding the nuclear localization
10 signal of SV40 large T antigen.

B. pRS316/HIS3:TetR/Term

The construction protocol for this plasmid was the same as described above for subcloning a *HIS3* DNA into pRS306/Term except that the vector for subcloning was pRS316/Term described above.

15 C. pRS306/1xLexAop/HIS3:TetR

Oligonucleotides LexAop (100a) and LexAop (100b) containing a single copy of LexA operator were phosphorylated with *T4* polynucleotide kinase (Gibco BRL, Grand Island, NY) at 37°C for one hour.

20 LexAop (100a) SEQ ID NO: 18
5'-AATTGCTCGAGTACTGTATGTACATACAGTAG

LexAop (100b) SEQ ID NO: 19
5'-AATTCTACTGTATGTACATACAGTACTCGAGC

Following phosphorylation, the oligonucleotides were annealed by heating at 70°C for 10 minutes followed by cooling to room temperature. The annealed
25 oligonucleotide containing 5' and 3' *EcoRI* overhanging ends was subcloned into pRS306/HIS3:TetR/Term previously digested with *EcoRI*. The number

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of copies of inserted oligonucleotide was confirmed by DNA sequencing. The plasmid containing a single copy of the LexA operator was designated pRS306/1xLexAop/HIS3:TetR.

D. pRS316/2xLexAop/HIS3:TetR

5 The subcloning protocol for this construct was the same as described above for pRS306/1xLexAop/HIS3:TetR. The annealed oligonucleotides encoding the LexA operator included overhanging *Eco*RI ends and during ligation, the individual annealed fragments were able to multimerize, inserting into the parental plasmid more than one copy of the
10 desired LexA sequence. The number of copies of inserted oligonucleotides was confirmed by DNA sequencing.

E. pRS306/2xLexAop/HIS3:TetR

A DNA fragment containing two copies of LexA operator and the chimeric *HIS3*:TetR reporter was excised from pRS316/2xLexAop/*HIS3*:TetR by digestion with *KpnI* and *BamHI* restriction enzymes. The fragment was gel-purified and subcloned into pRS306/Term previously digested with *KpnI* and *BamHI* and the resulting construct was sequenced to confirm the presence of two copies of the LexA operator.

20 F. pRS306/4xLexAop/HIS3:TetR
and pRS306/8xLexAop/HIS3:TetR

A pair of oligonucleotides SH101A and SH101B were utilized in PCR to amplify the LexA binding site multimer from the plasmid SH18-34ΔSpe [Hollenberg, S.M., *et al.*, *Mol. Cell. Biol.* **15**:3813-3822 (1995)].

SH101A SEQ ID NO: 20
25 5'-CCGGAATTCTCGAGACATATCCATATCTAATC

SH101B SEQ ID NO: 21
5'-CCGGAATTCACTAATCGCATTATCATC

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The amplification product containing four copies of LexA operator was gel-purified, digested with *Eco*RI, and subcloned into pRS306/HIS3:TetR/Term previously digested with *Eco*RI. The number of LexA operators were determined by DNA sequencing.

5 G. pRS306/8xLexAop/HIS3::TetR

A PCR strategy was used to link the 5' promoter sequence of the yeast *HIS3* gene encompassing nucleotides -75 to +23 to the translational start of *TetR*. Sequences encoding the SV40 large T antigen nuclear localization signal were fused in frame with the nucleotide sequence encoding the last amino acid residue of TetR. The PCR product was digested with *EcoRI* and *BamHI* and inserted into pRS306/Term previously digested with *EcoRI* and *BamHI*. The resulting plasmid was designated pRS306/HIS3:TetR/Term, and was shown to encode the complete TetR protein in frame with the nuclear localization signal of SV40 large T antigen. The fusion protein is followed by four amino acids generated by the vector backbone (Arg-Ile-His-Asp).

The LexA binding site multimer from the plasmid pSH18-34ΔSpe [Hollenberg, S.M. *et al.*, *Mol. Cell. Biol.* **15**:3813-3822 (1995)] was amplified by PCR, digested with *Eco*RI, and subcloned into the *Eco*RI site of pRS306/HIS3:TetR/Term resulting in plasmid pRS306/8xLexAop/TetR.

20 H. pADH/TetR

The DNA coding sequence of TetR was amplified by PCR from pSLF104 using two oligonucleotides, NcoI-TetR and 3'-TetR terminal primer (SEQ ID NO: 17).

25 NcoI-TetR SEQ ID NO: 22
5'-CATGCCATGGCCATGTCTAGATTAGATAAAAG

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The resulting product was gel-purified, digested with *Nco*I and *Bam*HI, and subcloned into a pBTM116 [Bartel, *et al.*, in Cellular Interactions in Development: a Practical Approach, Hartley (ed.), IRL Press; Oxford, pp. 153-179 (1993)] shuttle vector containing an ADH promoter, previously
5 digested with *Nco*I and *Bam*HI. For construction of this vector, DNA generated by PCR and DNA obtained by restriction enzyme digestion of the polylinker region in plasmid pBluescript (Stratagene, La Jolla, California) were used to engineer additional restriction sites 5' and 3' of the ADH promoter. The TetR protein encoded from this construct is expressed
10 containing additional amino acids Met⁻²-Ala⁻¹ before the initiating methionine and also contains the nuclear localization signal of SV40 large T antigen located after the last amino acid of TetR as described above.

I. pRS306/ADH:TetR/Term

A fragment encoding the ADH promoter and TetR was removed
15 from plasmid pADH/TetR with *Xho*I and blunted-ended with the large fragment of DNA polymerase I (Gibco BLR, Grand Island, NY). *Eco*RI linkers (New England BioLabs, Beverly, MA) were added and the fragment was digested with *Eco*RI and *Bam*HI. The resulting fragment was gel-purified and ligated into pRS306/Term previously digested with *Eco*RI and *Bam*HI.

20 J. pRS306/4xLexAop/ADH::TetR
and pRS306/8xLexAop/ADH::TetR

The subcloning protocol used to insert multiple copies of the LexA operator into pRS306/ADH:TetR/Term was the same as described
previously for pRS306/4xLexAop/HIS3:TetR and
25 pRS306/8xLexAop/HIS3:TetR.

III. Plasmids Encoding Binding Proteins

A. pLexA-CBD

A DNA fragment containing the CREB binding domain of CBP (CBD), amino acids 461-682, was PCR amplified from plasmid CBP-0.8 [Chrivia, J.C. *et al.*, *Nature* **365**:855-859 (1993)] using a pair of oligonucleotides designated 5' CBD primer and 3' CBD primer.

5' CBD primer SEQ ID NO: 23
5'-GCGAATTCGCCAGGGCAACAGAATGCCACT

3' CBD primer SEQ ID NO: 24
5'-CGGGATCCTGGCTGGTTACCCAGGATGCCTTG

Following gel purification, the amplification product was digested with *EcoRI* and *BamHI*, and ligated into plasmid pBTM116 [Bartel, *et al.*, in *Cellular Interactions in Development: a Practical Approach*, (ed) Hartley, D.A. (IRL Press, Oxford), pp. 153-179 (1993)] previously digested with *EcoRI* and *BamHI*.

B. pVP16-CBD

A DNA fragment encoding the CBP sequence was excised from pLexA-CBD by digestion with *EcoRI* and *BamHI*. Plasmid pLexA-CBD was linearized with *EcoRI* digestion, the resulting overhanging ends blunt-ended using the Klenow fragment of DNA polymerase I, and the ends ligated with *BamHI* linkers. The resulting fragment was inserted into pVP16 [Hollenberg, *et al.*, *Mol. Cell. Biol.* **15**:3813-3822 (1995)] previously digested with into *BamHI*.

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C. pVP16 CREB

Plasmid pcDNA3/CREB283 [Sun and Maurer, *J. Biol. Chem.* 270:7041-7044 (1995)], containing the VP16 transactivation domain fused to sequences of the rat CREB transactivation domain (1 to 283 aa) was linearized with *Xho*I and *Bam*HI linkers (New England BioLab) ligated to the resulting blunt-ended *Xho*I sites. DNA encoding the VP16/CREB chimeric protein was removed with *Hind*III and *Bam*HI digestion and following gel purification, ligated into the *Hind*III and *Bam*HI sites of pVP16 which encodes the *LEU2* gene.

10 D. pVP16-CREB(BglII-SacII)-LacZ

A DNA fragment encoding β -galactosidase was PCR amplified from plasmid pSV- β -galactosidase vector (Promega, Madison, WI) using a pair of oligonucleotides, 5' β -gal primer and 3' β -gal primer and inserted into the *NorI* site of pVP16 to produce pVP16-LacZ.

15 5' β -gal primer SEQ ID NO: 29
5'-ATGGTACCAGCGGCCGCTAGTCGTTTTACAACGTCGTGAC

3' β -gal primer SEQ ID NO: 30
5'-ATGGTACCGCGGCCGCTTATTTTGGACACCAGACCAAC

A PCR fragment containing CREB sequences encoding amino acid residues 1 to 283 was amplified from plasmid pRSV-CREB341 [Kwok, *et al.*, *Nature* **380**: 642-646 (1996)] using a pair of oligonucleotides, 5' CREB 341 primer and 3' CREB 283 primer, and inserted into pVP16-LacZ vector at the *Bam*HI site.

25 5' CREB 341 primer SEQ ID NO: 25
5'-CGCGGATCCGGATGACCATGGACTCTGGAG

3' CREB 283 primer SEQ ID NO: 28
5'-CGCGGATCCGTGCTGCTTCTTCAGCAGGCTG

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To generate a cassette vector for producing and subcloning mutated CREB sequences as described below, PCR was used to engineer a *Bgl*III site using oligonucleotides 5' *Bgl*III primer and 3' *Bgl*III primer, at nucleotides 273 to 278 and a *Sac*II site using oligonucleotides 5' *Sac*II primer and 3' *Sac*II
5 primer at nucleotides 500 to 505 of the CREB activation domain.

5' *Bgl*III primer SEQ ID NO: 31
5'-CGGAGATCTAAAGAGACTTTTCTCCGGAAGCTCAG

3' *Bgl*III primer SEQ ID NO: 32
5'-CGGAGATCTTTACAGGAAGACTGAACTGT

10 5 SacII primer SEQ ID NO: 33
 5'-CCACCGCGGCAGTGCCAACCCCGATTTC

3' *Sac*II primer SEQ ID NO: 34
3'-CATCCGCGGTGGTGATGGCAGGGGCTGA

E. pLexA-CREB 283

15 A DNA fragment containing the rat CREB transactivation domain (amino acids 1 to 283) was excised from pcDNA/CREB283 [Sun and Maurer, *supra*] with *Sma*I and *Xba*I digestion. The 5' *Xba*I site was blunt ended with the large fragment of DNA polymerase I (Gibco BRL, Grand Island, NY) and *Sal*I linkers (New England Biolabs, Beverly, MA) added.

20 The fragment was digested with *Sal*I and subcloned into the *Sal*I site of pBTM116.

F. pLexA-CREB 341

A DNA fragment containing the rat CREB 341 cDNA was amplified by PCR from pcDNA/CREB341 [Kwok, *supra*] using a pair of oligonucleotides, 5' CREB 341 primer (SEQ ID NO: 25) and 3' CREB 341 primer.

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3' CREB 341 primer SEQ ID NO: 26
5'-CGCGGATCCTTAATCTGACTTGTGGCAGTA

After gel purification, the PCR product was digested with *Bam*HI, and subcloned into the *Bam*HI site of pBTM116.

5 G. pLexA-CREB 341-M1

A DNA fragment containing the rat CREB sequence with a mutation changing serine at position 133 to alanine was amplified by PCR from plasmid Rc/RSV CREB-M1 [Kwok, *et al.*, *supra*] using the same set of primers as described for pLexA-CREB 341, 5' CREB 341 primer (SEQ ID NO: 25) and 3' CREB 341 primer (SEQ ID NO: 26). The resulting amplification product was gel-purified, digested with *Bam*HI, and subcloned into the *Bam*HI site of pBTM116.

H. pVP16-CREB M1

A PCR fragment containing CREB sequences coding for amino acid residues 1 to 283 including the serine 133 mutation to alanine was amplified using a pair of oligonucleotides, 5' CREB 283 primer and 3' CREB 283 primer (SEQ ID NO: 28). The PCR fragment was gel-purified, digested with *Bam*HI and inserted into the *Bam*HI site of pVP16.

20 5' CREB 283 primer SEQ ID NO: 27
5'-CGCGGATCCCCATGACCATGGAATCTGGAGCC

I. pLexA-SRF

A DNA fragment containing human SRF was excised from plasmid pCGN-SRF [Grueneberg, D.A., *et al.*, *Science*, **257**:1089-1095 (1992)] with *Xho*I and *Bam*HI digestion. The *Xho*I site of the fragment was blunt-ended by the large fragment of DNA polymerase I (Gibco BRL, Grand Island, NY), ligated with *Bam*HI linkers, digested with *Bam*HI, and inserted

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into pBTM116 previously digested with *Bam*HI.

J. pVP16-Tax

A DNA sequence encoding full length Tax protein was excised from pS6424 [Kwok, R.P.S., *et al.*, *Nature* **380**:642-646 (1996)] with *Bam*HI
5 digestion and was inserted into pVP16 previously digested with *Bam*HI.

IV. Plasmids For Binding Protein Controls

A. pLeu

Plasmid pVP16 was digested with *Hind*III and *Bam*HI to remove the fragment encoding the VP16 transactivation domain. The digested
10 vector was blunt-ended and self-ligated.

B. pLexA-VP16

The VP16 transactivation domain was PCR amplified from pGal-VP16 [Sadowski, *et al.*, *Nature* **335**:563-564 (1988)] with a pair of oligonucleotides, 5'-VP16SH and 3'-VP16SH and the resulting amplification
15 product was digested with *Cla*I, blunt-ended, and inserted into pBTM116.

5'-VP16SH SEQ ID NO: 35
GGCTATCGATACGGCCCCCGACCGAT

3'-VP16SH SEQ ID NO: 36
GCGTATCGATCTACCCACCGTACTCGTC

20 C. pLexA-Lamin

See Hollenberg, S.M. *et al.*, *Mol.Cell.Biol.* **15**:3813-3822 (1995)].

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V. Plasmids Encoding Reporter Gene Controls

A. pRS306/Term

The alcohol dehydrogenase (ADH) terminator sequence was excised from plasmid pBTM116 [Bartel, *et al.*, in *Cellular Interactions in Development: a Practical Approach*, (ed) Hartley, D.A. (IRL Press, Oxford), pp. 153-179 (1993)] with *Sph*I and *Pst*I restriction enzymes and both 3'-overhanging sequences were blunted by *T4* DNA polymerase (Gibco BLR, Grand Island, NY). The fragment was gel-purified and subcloned into the blunt-ended *Not*I site in pRS306 [Sikorski and Hieter, *Genetics*:122:19-27 (1989)]. The orientation of inserted fragment was determined by DNA sequencing.

B. pRS316/Term

The subcloning protocol for inserting the ADH terminator sequence into pRS316 was the same as described for inserting the ADH sequence in pRS306.

Example 2

Generation of Yeast Assay Transformant

Selection of an appropriate yeast assay strain is an empirical determination based on growth characteristics of the transformed alternatives. A general method to make the appropriate selection is described as follows.

Candidate yeast assay strains were transformed individually with reporter gene constructs and/or a plasmid encoding one of the experimental binding proteins. Assay strains thus transformed were then compared for relative differences in growth characteristics, with an optimal assay strain showing negligible growth on media lacking histidine and vigorous growth on media containing histidine. In practical application of this first step in selection using various plasmids transformed into assay strain YI584, the following results were observed.

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When the plasmid pLexA-VP16 encoding both the LexA DNA binding domain and the VP16 transactivating domain as a single protein was introduced into the assay cells, growth in the absence of histidine in the media was significantly reduced three days after transformation.

5 In assays including transformation with plasmids encoding multiple copies of the *tet* operator upstream of the *HIS3* gene, the following plasmids were separately utilized:

pRS303/1xtetop-*HIS* (encoding a single *tet* operator sequence),
pRS303/2xtetop-*HIS* (encoding two *tet* operator sequences),
10 pRS303/3xtetop-*HIS* (encoding three *tet* operator sequences),
pRS303/4xtetop-*HIS* (encoding four *tet* operator sequences),
pRS303/6xtetop-*HIS* (encoding six *tet* operator sequences),
pRS303/8xtetop-*HIS* (encoding eight *tet* operator sequences), or
pRS303/10xtetop-*HIS* (encoding ten *tet* operator sequences).

15 In the assay strains transformed with plasmids encoding either one, two, or three copies of the *tet* operator upstream from the *HIS3* gene, cells grew on media lacking histidine at a rate similar to cells grown on media containing histidine. In yeast assay strains transformed with plasmids encoding either six, eight, or ten copies of the *tet* operator upstream from the *HIS3* gene, cell
20 growth was low suggesting that these strains would not be useful in assays to examine binding and interruption of binding between test proteins. These results suggested that, in assay strains transformed with a reporter plasmid having more than three *tet* operator sequences upstream from the *HIS3* gene, normal activity of the *HIS3* promoter is disrupted and that these plasmids
25 would not be useful.

In assays wherein yeast cells were transformed with only reporter plasmids (and not plasmids encoding binding partner fusion proteins) encoding multiple copies of the LexA operator 5' of the TetR gene, the

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following results were observed. Growth of assay cells transformed with plasmids bearing one, two, four, and eight copies of the regulatory LexA operator upstream of the TetR gene appeared to be "copy number" dependent. Yeast cells transformed with plasmids having two copies of the LexA operator
5 grew at a rate significantly higher than those assay cell transformed with a plasmid bearing only one copy of the operator. Cells transformed with plasmids encoding either four or eight LexA operators upstream of the TetR gene grew at an approximately equal rate, and better than assay cells bearing a TetR gene driven by two copies of the operator.

10 When the alcohol dehydrogenase (ADH) promoter was included upstream of the LexA operator (plasmids encoding either four or eight LexA operators) in the various reporter gene constructs, cell viability was the lowest.

The various cell lines constructed by the methods described
15 above are shown in Table 1, wherein various transformed yeast strains are identified (Strain #) along with the number of LexA operator sequences in the plasmid encoding TetR, the number of tetracycline operator sequences regulating expression of HIS3, and relative growth rate of the transformed strain on media containing histidine. It is important to note that growth
20 variation of transformed cells in media containing histidine is observed, even in cell lines identically transformed. The number of "+" signs in Table 1 is indicative of the host cell's relative ability to grow on media lacking histidine in the absence of transformation with plasmids encoding potential binding proteins. Also in Table 1, a subscript "a" is indicative of transformation with
25 a plasmid bearing the alcohol dehydrogenase promoter; absence of a subscript "a" indicates use of the HIS3 promoter.

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Table 1
Various Yeast Transformants

5	Diploids L40				Diploids L40				
	Strain #	LexA	TetOp	His+	Strain #	LexA	TetOp	His+	
	YI579	1X	2X	+++	YI602	4X _n	6X	--	
	YI581	1X	2X	+++	YI607	4X	6X	+++	
					YI628	4X	6X	+++	
	YI580	2X	2X	+++	YI632	4X _n	6X	- ?	
	YI582	2X	2X	+++					
10	Diploids L40				YI605	4X _n	10X	--	
	Strain #	LexA	TetOp	His+	YI610	4X	10X	+	
	YI583	4X	2X	+++	YI622	4X	10X	++	
	YI585	4X	2X	+++	YI626	4X _n	10X	++	
	YI587	4X	2X	+++					
	YI589	4X	2X	+++	YI592	8X	2X	+++	
15					YI596	8X _n	2X	+++	
	YI584	8X	2X	+++	YI598	8X	4X	+	
	YI586	8X	2X	+++	YI635	8X _n	4X	+	
	YI588	8X	2X	+++	YI637	8X	4X	++	
	YI590	8X	2X	+++	YI601	8X	6X	+	
20	Diploids L40				YI608	8X _n	6X	+	
	Strain #	LexA	TetOp	His+	YI629	8X _n	6X	+++	
	YI591	2X	2X	+++	YI631	8X	6X	+++	
	YI594	2X	2X	+++					
	YI597	2X	4X	--	YI604	8X	10X	+	
25	YI633	2X	4X	+	YI611	8X _n	10X	+	
	YI636	2X	4X	++	YI623	8X _n	10X	++	
					YI625	8X	10X	++	
	YI600	2X	6X	--					
	YI606	2X	6X	+	Strain#	LexA	TetOp	strain	His+
	YI630	2X	6X	+	YI664	4X _n	3X	w303(50)	+++
	YI627	2X	6X	+++	YI666	4X _n	3X	w303(51)	+++
30					YI668	4X _n	2X	L40 (69)	+++
	YI603	2X	10X	+	YI670	4X _n	2X	L40 (70)	+++
	YI621	2X	10X	++					
	YI609	2X	10X	+	YI665	8X _n	3X	w303(50)	+++
	YI624	2X	10X	++	YI667	8X _n	3X	w303(51)	+++
35					YI671	8X _n	3X	L40 (69)	+++
	YI593	4X _n	2X	+++	YI669	8X _n	2X	L40 (69)	+++
	YI595	4X	2X	+++	YI671	8X _n	2X	L40 (70)	+++
	YI599	4X _n	4X	--	YI671	8X _n	6X	L40 (69)	+++
	YI634	4X	4X	+					
	YI638	4X _n	4X	+					

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Example 3 CREB/CBP Binding Interaction

Use of the split-hybrid assay for studies of protein/protein binding wherein one of the binding components is randomly mutagenized was carried out using CREB and CBP binding proteins. The binding of CREB to CBP has been shown to require the phosphorylation of the CREB serine residue at position 133 in a region designated the "kinase-inducible domain" (KID) [Chrivia, *et al.*, *Nature* **365**, 855-859 (1993); Kwok, *et al.*, *Nature* **370**, 223-226 (1994)]. Functionally, changing serine at position 133 to alanine (a mutant designated CREB-M1) abolishes the ability of CBP to activate CREB-mediated transcription. Preliminary studies have indicated that the CREB-M1 mutant in the split-hybrid system prevents the interaction with CBP and subsequent growth of the yeast assay strain on media lacking histidine. Precisely what other portions of the KID of CREB are required for binding to CBP is unknown, however. To define other potentially important amino acid residues, the KID (amino acid residues 102 to 160) of CREB 341 was randomly mutagenized using PCR.

A. PCR Mutagenesis and Creation of Mutant Library

The technique used for mutagenic PCR was a modification of that described by Uppaluri and Towle [*Mol. Cell. Biol.* **15**, 1499-1512 (1995)]. The reaction mixture contained 20 ng of pVP16-CREB(BglIII-SacII)-LacZ, 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, pH 8.8, 6.1 mM MgCl₂, 0.5 mM MnCl₂, 6.7 μM EDTA, 10 mM β-mercaptoethanol, 1 mM primers, 1mM each dGTP, dTTP, and dCTP, 400 μM dATP, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). After seven cycles of PCR (94°C for 40 sec, 50°C for 40 sec, and 72°C for 40 sec), the PCR product was amplified a second time using the same primers and *Vent* DNA polymerase (New England BioLabs, Beverly, MA) under the same conditions for 25 cycles. The resultant PCR product was gel purified, digested with *Bgl*III and

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SacII, and inserted into the *BglIII* and *SacII* sites of pVP16-CREB(*BglIII*-*SacII*)-LacZ (construction of which is described above). The resulting plasmids were transformed into DH5 α bacterial cells. Transformants were pooled and plasmid DNA was isolated by CsCl gradient centrifugation.

5 B. Construction and Use of pVP16-CREB(*BglIII*-*SacII*)-LacZ

 A DNA fragment encoding the β -galactosidase gene was fused in frame to the carboxyl-terminal end of VP16-CREB as described above. The carboxy-terminal tag allowed identification of clones that contain frame-shift and nonsense mutations; colonies that remain positive for β -galactosidase
10 were presumed to contain an open reading frame throughout the mutated region. To facilitate the subcloning of mutated sequences, a cassette version of the CREB cDNA was generated that contained *BglIII* and a *SacII* sites flanking the 5' and 3' ends of the KID, respectively. These modifications altered the amino acid residue at position 168 from valine to alanine. The
15 cDNA altered in this manner was indistinguishable from the original VP16-CREB and from VP16-CREB-LacZ when tested in the split hybrid assay. Primers complementary to regions flanking the KID were used in mutagenic PCR amplification reactions as described above under conditions which were optimized to achieve one to three mutations in the 177 bp region encoding the
20 KID. PCR products were introduced into pVP16-CREB(*BglIII*-*SacII*)-LacZ in place of wild-type sequence. A library of mutated sequences was transformed into yeast assay strain YI584 expressing LexA-CBD. Approximately 27,000 yeast transformants were screened, yielding about 5,000 colonies that were capable of growing on selective media supplemented with 10 μ g/ml of tetracycline and 1 mM of 3AT, determined as described below.
25

 Two screening steps were performed to eliminate uninformative mutations and false positives. First, filter β -galactosidase assays were performed by standard methods [Vojtek, *et al.*, *Cell* 74:205-214 (1993)] on the 5,000 colonies which exhibited positive growth on media lacking

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tryptophan, histidine, uracil, leucine, and lysine to eliminate expressed proteins having frame-shift and nonsense mutations. Five hundred thirty six colonies developed a dark blue color, whereas 412 colonies turned white and were presumed to express mutants containing either frame-shift or nonsense mutations. The other colonies developed a pale blue color, and control experiments suggested that these colonies may have expressed unstable lacZ fusion proteins. Pale blue colonies were not analyzed further.

DNA from 536 dark blue colonies was isolated and transformed into *E.coli* MC1066 cells. One hundred ninety three pVP16-CREB-(BgIII-SacII)-LacZ cDNAs were then isolated.

In a second screening step, the 193 cDNAs were separately re-transformed along with pLexA-CBD into the split-hybrid strain as well as into the two-hybrid L40 strain [Vojtek, *et al.*, *supra*] in order to identify false positives and confirm that the mutant CREB proteins did not interact with CBP. Among the 193 cDNAs re-screened, 152 did not interact with CBP in the yeast two-hybrid system, 15 interacted weakly, and 26 interacted like wild type CREB.

Following these two screening steps, the 152 CREB mutants were sequenced. Seventy CREB mutants were found to contain a single amino acid change. Sixty four CREB mutants contained two amino acid residue mutations and 13 mutants contained more than two amino acid mutations. Mutants containing more than one amino acid alteration were not analyzed further. The expression level of mutant proteins having one amino acid change were determined using a standard β -galactosidase assay.

The CREB mutations identified in the split-hybrid screen were shown to carry amino acid changes centered around the phosphorylation site at serine at position 133. No disrupting mutations were found to contain amino acid alterations outside of the region between amino acids 130 to 141. Most of the mutations abrogated the PKA phosphorylation region, but others were identified at isoleucine position 137, leucine at position 138, and leucine

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at position 141. The mutations at positions 137, 138, and 141 generally changed the hydrophobic residues at these positions to polar residues. The ability of the split-hybrid system to detect only a limited number of CREB mutants, many of which have been proposed previously to disrupt CREB
5 association with CBP [Parker, *et al.*, *Mol. Cell. Biol.* **16**, 694-703. (1996)], indicates the specificity of the split-hybrid system.

These results lead to interesting suggestions. Various CREB mutations were identified which disrupt CREB-CBP interaction and the majority of disrupting mutations occurred in the CREB PKA phosphorylation
10 motif. This result was consistent with previous observations that nonphosphorylated CREB and CBP do not interact [Kwok, *et al.*, *Nature* **370**:223-226 (1994)]. The most common motif for PKA phosphorylation is an RRX(S/T)X amino acid sequence but RX(S/T)X and KRXX(S/T)X are also phosphorylated [Kemp and Pearson, *T.I.B.S.* **15**, 342-346 (1990)]. The
15 arginine residues in the phosphorylation site are critical for electrostatic interactions with acidic amino acid residues in the catalytic subunit of PKA [Knighton, *et al.*, *Science* **253**, 414-420 (1991)], and consistent with this observation, CREB mutants with changes at arginine residues 130 and 131 were identified in the split hybrid assay that did not interact with CBP.

20 Results also showed that CREB mutations at amino acids proline at residue 132 and tyrosine 134 were unable to bind CBP. It is likely that the mutations at these residues adversely affect the structure of the phosphorylation motif, although these positions are generally thought to be less critical to CBP binding. It is possible that the substitution of proline at
25 position 132 with threonine created a new phosphorylation site (RXTX) that interfered with the critical phosphorylation of serine at position 133. Although not generally thought to be part of the "classical" consensus PKA phosphorylation motif, hydrophobic amino acids are commonly found carboxy-terminal to PKA sites [Kemp, *et al.*, *T.I.B.S.* **19**:440-444 (1994)].
30 The importance of these flanking residues may explain the frequent occurrence

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of disrupting mutations involving tyrosine at position 134. Further studies will be directed to determining if mutations of proline at position 134 and tyrosine at position 134 directly disrupt phosphorylation of serine at position 133 or disrupt binding of CREB to CBP by some other mechanism.

5 In addition, substitution of serine at position 133 with threonine also prevented the interaction of CREB and CBP. PKA protein substrates containing a phosphorylatable threonine residue are known to exist in nature (*i.e.*, protein phosphatase inhibitor 1 and myelin basic protein), although they are less common than those with phosphorylatable serines [Zetterqvist, *et al.*,
10 in Peptides and Protein Phosphorylation, (ed.) Kemp, B.E. (CRC Press, Boca Raton, FL), pp. 172-187 (1990)], and synthetic peptides containing serine to threonine substitutions are relatively poor substrates for PKA phosphorylation [Zetterqvist, *et al.*, *supra*]. In the split-hybrid assay, however, it is unclear whether the mutation of threonine at position 133 disrupts the CREB-CBP
15 interaction or if the mutant fails to become phosphorylated. Despite previous observations that serine residue at position 133 of mammalian CREB can be phosphorylated by a variety of protein kinases other than PKA, for example calcium/calmodulin-dependent protein kinase II and IV, protein kinase C, and a nerve growth factor (NGF)-activated CREB kinase [Sheng, *et al.*, *Neuron*
20 4:571-582 (1990); Sheng, *et al.*, *Science* 252:1427-1430 (1991); Xie and Rothstein, *J. Immunol.* 154:1717-1723 (1995); Ginty, *et al.*, *Cell* 77:1-20 (1994)], it is not known which, if any, of these particular protein kinases are able to phosphorylate CREB at the serine at position 133 in yeast. The requirement for integrity of the entire RRXSX amino acid sequence, however,
25 suggests that PKA is a reasonable candidate.

 The second category of mutations were identified adjacent the PKA phosphorylation motif. Amino acids isoleucine at position 137 and leucine at position 138 have previously been suggested to be important for hydrophobic interactions of CREB with CBP [Parker, *et al.*, *Mol. Cell. Biol.*
30 16, 694-703 (1996)]. In this study, most of the mutations at position 137 and

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138 converted these hydrophobic residues to polar amino acids. Thus, another possibility for the failure of these mutants to bind to CBP is that changes at these positions affect protein folding. Similarly, the mutation at position 141 substituted a polar residue for the wild-type hydrophobic leucine, and this
5 mutation also has the potential to affect protein folding.

Substitution of the isoleucine at position 137 with a hydrophobic phenylalanine residue was found to disrupt the interaction between CREB and CBP as well. This result could have been the result of a detrimental effect on folding because of the steric hindrance associated with the comparatively
10 larger size of phenylalanine. Alternatively, the proposed hydrophobic interactions between CREB and CBP are somewhat specific. Structural studies will be directed to definitively determine how these mutations affect binding.

Perhaps most surprising was the finding that critical mutations
15 were restricted to a small region in the KID sequence, even though the relatively low affinity of phosphorylated CREB and CBP, determined to be between 250 and 400 nM by fluorescence anisotropy measurements [Kwok, *et al.*, *Nature* **370**, 223-226 (1994)], is consistent with a restricted protein binding domain. The capability of the split-hybrid system to screen for a
20 limited number of CREB mutants suggests that the system is highly specific, and thus, should be useful to identify mutations which disrupt interacts between other pairs of binding proteins.

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Example 4 **Tax/SRF Binding Interaction**

To further investigate the feasibility of using the split-hybrid system to study protein-protein interactions, a pair of well characterized
5 interacting proteins, SRF and Tax, was tested. Previous studies indicated that SRF and Tax interact in a standard yeast two-hybrid system suggesting that the proteins may be utilized in the split hybrid assay. Plasmid pLexA-SRF, containing a human SRF cDNA fused to the LexA DNA binding domain, was transformed into strain YI584 along with either pVP16-Tax or pVP16 alone.
10 As with the pLexA-VP16 transformation, the yeast strains co-expressing LexA-SRF and VP16-Tax failed to yield any colonies on medium lacking histidine. In contrast, when LexA-SRF was co-transformed with a vector encoding the VP16 activation domain alone, yeast growth occurred on medium lacking histidine, suggesting that TetR expression was not activated. These
15 results demonstrated that a protein-protein interaction in the split-hybrid system can effectively prevent yeast growth and further indicated the utility of the assay for the study of various protein/protein interactions.

Example 5 **Casein Kinase Binding Assays**

20 Hrr25

In another example of use of the split hybrid assay to examine protein/protein interactions, Hrr25, a yeast casein kinase isoform, or human casein kinase I isoform δ , was employed in the assay with a known binding partner protein.

25 Previous work using the two hybrid assay had identified three genes encoding proteins which interact with the yeast casein kinase isoform Hrr25. Proteins encoded by the genes were designated TIH1, TIH2, and TIH3. The Hrr25 expression construct which was generated for use in the two hybrid assay was used in combination with the individual TIH encoding
30 constructs in the split hybrid assay to determine if interaction between the

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binding partners would decrease growth of assay yeast cells on media lacking histidine. Construction of the Hrr25 expression plasmid and isolation of plasmids encoding TIH proteins is discussed below.

In order to identify genes encoding proteins that interact with
5 *S. cerevisiae* HRR25 CKI protein kinase, a plasmid library encoding fusions between the yeast GAL4 activation domain and *S. cerevisiae* genomic fragments ("prey" components) was screened for interaction with a DNA binding domain hybrid that contained the *E. coli* *lexA* gene fused to HRR25 ("bait" component). The fusions were constructed in plasmid pBTM116
10 which contains the yeast TRP1 gene, a 2 μ origin of replication, and a yeast ADHI promoter driving expression of the *E. coli* *lexA* protein containing a DNA binding domain (amino acids 1 to 202).

Plasmid pBTM116::HRR25 encoding the *lexA*::*HRR25* fusion protein was constructed in several steps. The DNA sequence encoding the
15 initiating methionine and second amino acid of HRR25 was changed to a *Sma*I restriction site by site-directed mutagenesis using a MutaGene mutagenesis kit from BioRad (Richmond, California). The DNA sequence of HRR25 is set out in SEQ ID NO: 39. The oligonucleotide used for the mutagenesis is set forth below, wherein the *Sma*I site is underlined.

20 5'-CCTACTCTTAGGCCCGGGTCTTTTTAATGTATCC-3'
(SEQ ID NO: 37)

After digestion with *Sma*I, the resulting altered HRR25 gene was ligated into plasmid pBTM116 at the *Sma*I site to create the *lexA*::HRR25 fusion construct.

25 Interactions between bait and prey fusion proteins were detected in yeast reporter strain CTY10-5d (genotype=*MATa ade2 trp1-901 leu2-3,112 his 3-200 gal4 gal80 URA3::lexA op-lacZ*.) [Luban, *et al.*, *Cell* 73:1067-1078 (1993)] carrying a *lexA* binding site that directs transcription of

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lacZ. Strain CTY10-5d was first transformed with plasmid pBTM116::HRR25 by lithium acetate-mediated transformation [Ito, *et al.*, *J.Bacteriol.* 153:163-168 (1983)]. The resulting transformants were then transformed with a prey yeast genomic library prepared as GAL4 fusions in the plasmid pGAD [Chien, *et al.*, *Proc.Natl.Acad.Sci (USA)* 21:9578-9582 (1991)] in order to screen the expressed proteins from the library for interaction with HRR25. A total of 500,000 double transformants were assayed for β -galactosidase expression by replica plating onto nitrocellulose filters, lysing the replicated colonies by quick-freezing the filters in liquid nitrogen, and incubating the lysed colonies with the blue chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal). β -galactosidase activity was measured using Z buffer (0.06 M Na_2HPO_4 , 0.04 M NaH_2PO_4 , 0.01 M KCl, 0.001 M MgSO_4 , 0.05 M β -mercaptoethanol) containing X-gal at a concentration of 0.002% [Guarente, *Meth. Enzymol.* 101:181-191 (1983)]. Reactions were terminated by floating the filters on 1M Na_2CO_3 and positive colonies were identified by their dark blue color.

Library fusion plasmids (prey constructs) that conferred blue color to the reporter strain co-dependent upon the presence of the HRR25/DNA binding domain fusion protein partner (bait construct) were identified. The sequence adjacent to the fusion site in each library plasmid was determined by extending DNA sequence from the GAL4 region. The sequencing primer utilized is set forth below.

5'-GGAATCACTACAGGGATG-3' (SEQ ID NO: 38)

DNA sequence was obtained using a Sequenase version II kit (US Biochemicals, Cleveland, Ohio) or by automated DNA sequencing with an ABI373A sequencer (Applied Biosystems, Foster City, California).

Four library clones were identified and the proteins they encoded are designated herein as TIH proteins 1 through 4 for Targets Interacting with

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HRR25-like protein kinase isoforms. The TIH1 portion of the TIH1 clone insert corresponds to nucleotides 1528 to 2580 of SEQ ID NO: 40; the TIH2 portion of the TIH2 clone insert corresponds to nucleotides 2611 to 4053 of SEQ ID NO: 41; and the TIH3 portion of the TIH3 clone insert corresponds to nucleotides 248 to 696 of SEQ ID NO: 42. Based on DNA sequence analysis of the TIH genes, it was determined that TIH1 and TIH3 were novel sequences that were not representative of any protein motif present in the GenBank database (July 8, 1993). TIH2 sequences were identified in the database as similar to a yeast open reading frame having no identified function. (GenBank Accession No. Z23261, open reading frame YBL0506)

When the various TIH proteins were used in the split hybrid assay in combination with Hrr25, it was observed that Hrr25/TIH3 binding, previously determined to be weaker than Hrr25/TIH2 or Hrr25/TIH1 interactions, produced the lowest level of growth in the transformed yeast strain.

CKI δ

In order to isolate cDNAs which encode proteins that interact with CKI δ , the two hybrid assay was performed using a LexA-CKI δ fusion protein as bait. The coding region of CKI δ was subcloned into a *Bam*HI site of pBTM116 and transformed into a yeast strain designated CKI δ /L40 (MAT a his3 Δ 200 trp1-901 leu2-3 112 ade2 LYS:: (lexAop)₄ HIS3 URA3:: (lexAop)₈-lcZ GAL 4). CKI δ /L40 was subjected to a large scale transformation with a cDNA library made from mouse embryos staged at days 9.5 and 10.5. Approximately 40 million transformants were obtained. Eighty-eight million were plated onto selective media lacking leucine, tryptophan and histidine. The ability of yeast transformants to grow in the absence of histidine suggested that there was an interaction between CKI δ and some library protein.

In a second screening, interaction of the two proteins was

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assayed by the ability of the interaction to activate transcription of β -galactosidase. Colonies that turned blue in the presence of X-gal were streaked onto media lacking leucine, tryptophan and histidine, grown up in liquid culture and pooled for isolation of total DNA. Isolated DNA was used
5 to transform *E. coli* strain 600 which lacks the ability to grow on media lacking leucine. Colonies that grew were used for plasmid preparation and three classes of cDNA were identified. One class was closely related to a *Drosophila* transcription factor dCREBa.

When CKI δ /CREB interaction was examined in the split hybrid
10 assay, cells were shown to grow on media containing histidine, but in the absence of histidine, growth was inhibited. Addition of small amounts of tetracycline to the cell culture restored the cell's ability to grow, suggesting that the interaction between CKI δ and CREBa was very weak.

Example 6 AKAP 79 Binding Assays

Expression Plasmid Utilized

In still another example of use of the split hybrid assay to examine protein/protein interactions, an anchoring protein for the cAMP dependent protein kinase, AKAP 79, was utilized separately with binding
20 partner proteins including the cAMP protein kinase regulatory subunit type I (RI), the cAMP dependent protein kinase regulatory subunit type II (RII) or calcineurin (CaN). Plasmids used in the assay were constructed as described below.

A 1.3 kb *NcoI*/*Bam*HI fragment containing the coding region
25 of AKAP 79 was isolated from a pET11d backbone and ligated into plasmid pAS1. Plasmid pAS1 is a 2 micron based plasmid with an ADH promoter linked to the Gal4 DNA binding subunit [amino acids 1-147 as described in Keegan et al., *Science*, 231:699-704 (1986)], followed by a hemagglutinin (HA) tag, polyclonal site and an ADH terminator. The expressed protein was

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therefore a fusion between AKAP 79 and the DNA binding domain of Gal4.

Plasmids encoding RI, RII or CaN were isolated from a pACT murine T cell library in a standard two hybrid assay using the AKAP 79 expression construct described above. Plasmid pACT is a leu2, 2 micron based plasmid containing an ADH promoter and terminator with the Gal4 transcription activation domain II [amino acids 768-881 as described in Ma and Ptashne, *Cell*, 48:847-853 (1987)], followed by a multiple cloning site. RI, RII and CaN encoding plasmids were isolated as described below.

A 500 ml SC-Trp yeast cell culture ($OD_{600} = 0.6-0.8$) was harvested, washed with 100 ml distilled water, and repelleted. The pellet was brought up in 50 ml LiSORB (100 mM lithium acetate, 10 mM Tris pH8, 1 mM EDTA pH8, and 1 M Sorbitol), transferred to a 1 liter flask and shaken at 220 rpm during an incubation of 30 minutes at 30°C. The cells were pelleted, resuspended in 625 μ l LiSORB, and held on ice while preparing the DNA.

The DNA was prepared for transformation by boiling 400 μ l 10 mg/ml salmon sperm DNA for 10 minutes after which 500 μ l LiSORB was added and the solution allowed to slowly cool to room temperature. DNA from a Mu T cell library was added (40-50 μ g) from a 1 mg/ml stock. The iced yeast cell culture was dispensed into 10 Eppendorf tubes with 120 μ l of prepared DNA. The tubes were incubated at 30°C with shaking at 220 RPM. After 30 minutes, 900 μ l of 40% PEG₃₃₅₀ in 100 mM Li acetate, 10 mM Tris, pH 8, and 1 mM EDTA, pH 8, was mixed with each culture and incubation continued for an additional 30 minutes. The samples were pooled and a small aliquot (5 μ l) was removed to test for transformation efficiency and plated on SC-Leu-Trp plates. The remainder of the cells were added to 100 ml SC-Leu-Trp-His media and grown for one hour at 30°C with shaking at 220 RPMS. Harvested cells were resuspended in 5.5 ml SC-Leu-Trp-His containing 50 mM 3AT (3-amino triazole) media and 300 μ l aliquots plated on 150 mm SC-Leu-Trp-His also containing 50mM 3AT. Cell were left to

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grow for one week at 30°C.

After four days, titer plates were counted and 1.1×10^5 colonies were screened. Large scale β -gal assays were performed on library plates and ten positive clones were isolated for single colonies. One of these colonies
5 grew substantially larger than the rest, and was termed clone 11.1. Sequence from clone 11.1 revealed an open reading frame 487 aa long which was correctly fused to the Gal-4 activation domain of pACT. The NIH sequence database was searched and the sequence was found to be closely homologous to the human calmodulin dependent protein phosphatase, calcineurin.

10 Additional screening using pACT Mu T-cell library DNA and the pASI AKAP 79 bait strain was performed in order to identify other AKAP 79 binding proteins by the protocol described above. Results from screening approximately 211,000 colonies gave one positive clone designated pACT 2-1. Sequencing and a subsequent data base search indicated that the clone had
15 91 % identity with rat type 1α regulatory subunit of protein kinase A (RI).

The library was rescreened using the same AKAP 79 bait and fifteen positives were detected from approximately 520,000 transformants. Of these fifteen, eleven were found to be homologous to the rat regulatory subunit type I of PKA. Each of these isolates were fused to the 5'
20 untranslated region of RI and remained open through the initiating methionine.

Split Hybrid Analysis

In split hybrid analysis of AKAP79 binding interactions, a plasmid was first constructed for expression of a LexA:AKAP 79 fusion protein. An AKAP 79 coding region was excised from pAS AKAP 79 as an
25 *NcoI/BamHI* fragment and inserted into pBTM116 previously digested with the same enzymes. The resulting plasmid was designated pBTM116-AKAP79.

Approximately 50,000 W303 yeast cells (strain YI665, see Table 1) in logarithmic growth were rinsed in media lacking histidine, suspended in 100 μ l to 200 μ l of the same media, and plated on agar lacking

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histidine (to select for absence of protein/protein interaction) and also lacking leucine and tryptophan (to select for transformants bearing expression constructs encoding AKAP 79 and its binding partner). When RII was employed as the AKAP 79 binding partner, 2 to 4 μ M tetracycline and 5 mM 3AT were required to prevent the transformed host from growing under conditions where the expressed proteins interacted.

Once conditions were established under which growth of the transformed host was eliminated, various candidate inhibitor compounds were separately added to the agar. It was presumed that if one of the candidate compounds was capable of disrupting AKAP 79 interaction with the binding partner protein, growth of the transformed host should be detectable in the vicinity of the compound on the agar. In the split hybrid assay wherein AKAP 79 and RII binding was examined, 2 μ l of a 30 mM stock solution of ICOS Compound 4273 in DMSO, 2 μ l of a 10 mM stock solution of ICOS Compound 1062 in DMSO, and 2 μ l DMSO alone (as a negative control) were spotted on to the plate which was incubated at 30°C for four to five days. For ICOS Compound 4273 a ring of growth was detected.

In order to determine an IC_{50} for an inhibitor identified as described above, alternative methods may be used. In one method, the inhibitor compound is added to the agar over a range of concentrations. Ideally, the compound is diluted to the point that host cell growth is essentially not detectable.

In another method, a 96 well plate is used and the compounds of interest are serially diluted across one row of a 96 well plate, one compound per row. Media lacking histidine, tryptophan, and leucine is added (presuming that the expression plasmids encoding the binding partners also encode trp and leu proteins) along with the appropriately transformed host yeast strain. Tetracycline and 3AT are added at concentration previously determined to extinguish growth of the transformed host cell. After two to five days incubation at 30°C, the plate wells are read at approximately 600

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nm using a plate reader. The concentration of inhibitor half way between zero and the lowest concentration that permits growth of the host cell to the level observed on media containing histidine is estimated to be IC₅₀.

A modification of this second method is particularly amenable
5 for use in a high throughput screen of large numbers of candidate inhibitors. For example, rather than attempting to determine the IC₅₀ for a previously identified inhibitor, separate candidate inhibitors are added to each well of a 96 well plate, preferably at more than one concentration, and host cell growth determined after several days incubation. Inhibitory activity of compounds
10 identified in this manner is confirmed on an agar plate and the IC₅₀ determined on 96 well plates, each assay as described above.

Example 7 **General Application of The Split-Hybrid Screen**

In order to examine general utility of the split hybrid system,
15 various experiments were conducted with binding proteins known to interact. In addition, a number of control experiments were included in order to determine if the effects observed with the known binding partners were in fact due to protein/protein interaction.

A. Yeast Assay Strain Construction

20 Yeast transformants used in assays indicated below were derived from LYS2-deficient strains AMR69 (Mat a *his3 lys2 leu2 trp1*, URA3:LexA::LacZ) and AMR70 (Mat α *his3 lys2 trp1 leu2*, URA3:LexA::LacZ) [Hollenberg, et al., *Mol. Cell. Biol.* **15**, 3813-3822 (1995); Chien, et al., *Proc. Natl. Acad. Sci. (USA)* **88**:97578-9582 (1991);
25 Fields and Song, *Nature* **340**:245-246 (1989)]. Yeast were grown in YEPD or selective minimal medium using standard conditions [Sherman, F., et al., Methods in Yeast Genetics, Cold Spring Harbor Lab., Cold Spring Harbor, NY (1986); *Methods in Enzymology*, Vol. 194 Guide to Yeast Genetics and

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Molecular Biology. Eds. Christine and Fink]. Derivatives of both AMR69 and AMR70 strains lacking URA3 were first generated by streaking cells on synthetic media containing 5 mg/ml 5-fluoro-orotic acid (5FOA) [*Methods in Enzymology*, Vol. 194 Guide to Yeast Genetics and Molecular Biology. Eds. Christine and Fink]. Two URA3 deficient mutants were required due to the fact that these strains were subsequently mated. URA3-deficient colonies were confirmed by testing for uracil auxotrophy and deletion of the URA::LexA::LacZ locus was confirmed by an absence of β -galactosidase activity assayed by standard methods. The mutant strains selected were designated 69-4 and 70-1.

Targeted integration of pRS306/8xLexAop/TetR was carried out by transforming [Hollenberg, *et al.*, *Mol. Cell. Biol.* **15**, 3813-3822 (1995)] the 69-4 strain with plasmid linearized at a unique *NcoI* site. The reporter gene construct was constructed using parental plasmid pRS306 which encodes URA3 as a selectable marker. Stably integrated plasmid thereby permitted selection on media lacking uracil. The positive uracil prototrophic strains were examined by Southern analysis to confirm insertion of the plasmid sequences.

Targeted integration of pRS303/2xtetop-LYS was carried out by transformation [Hollenberg, *et al.*, *supra*] of strain 70-1 with plasmid linearized at a unique *HpaI* site. The resulting lysine prototrophic strains were examined by Southern analysis to confirm insertion of the plasmid DNA.

The AMR69 derivative strain (MAT α) containing the pRS303/2xtetop-LYS insertion was mated with the AMR70-derivative strain (MAT a) containing pRS306/8xLexAop/TetR and mated cells were selected on media lacking both lysine and uracil. Single colonies were grown up and tested for the ability to grow on media lacking histidine. The resulting strain was designated YI584. In instances where yeast strains were transformed with other reporter gene pair combinations, the strains were uniquely designated.

Yeast bearing integrated reporter gene constructs were

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subsequently transformed [Hollenberg, *et al.*, *supra*] with plasmids encoding chimeric binding protein. Plasmids encoding the LexA DNA binding region were generally derived from parental plasmid pBTM116 which also encodes *TRP1* as a selectable marker. Plasmids encoding the VP16 transactivating domain were generally derived from parental plasmid pVP16 which also encodes *LEU2* as a selectable marker. Yeast cells which were successfully transformed with the four exogenous plasmids were therefore selected by an ability to grow on media lacking lysine, uracil, tryptophan, and leucine. Plasmids encoding various binding proteins were transformed into the yeast assay strain as indicated below.

B. Liquid Assay

After three days growth at 30°C on selection media as described above, a pool of colonies from each transformation was collected and diluted in 5 ml selective media. The mixture was vortexed and immediately sonicated for ten seconds. Cells in the resulting suspension were counted and seeded at 1000 cells/ml in selective media, 2 ml per 15 ml tube. Tetracycline, 3AT, and histidine were included as determined appropriate by the method described above. Each aliquot of cells was incubated with shaking for two days at 30°C and cell density measured at OD₆₀₀.

C. Characterization of the Assay

The utility of the split-hybrid assay was first determined using well characterized binding proteins and various controls.

In an initial study, YI584 cells were transformed with plasmids pLexA-VP16 and pLeu. While the expressed proteins from the two plasmids do not interact, pLexA-VP16 encodes a fusion protein containing the VP16 activation domain fused directly to LexA which contains a DNA binding domain. The chimeric LexA-VP16 protein is a strong transactivator for a promoter containing LexA operators. Plasmid pLeu is essentially a blank used

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as a control co-transformation plasmid.

Yeast transformed with the LexA-VP16 plasmid were able to express TetR protein as indicated by gel shift analysis using a *tet* operator oligonucleotide. In addition, the cells were unable to grow on media in the absence of histidine. Combined, these observations suggested that overexpressed TetR protein was capable of binding to *tet* operators and preventing the expression of *HIS3*. The transformed yeast grew on plates containing histidine, further indicating that overexpression of TetR did not have a toxic effect on the assay cells.

The results were consistent with previous observations and supported the earlier suggestion that activation of TetR expression, either through a single transcription factor or association of individual transcription factor domains, is capable of preventing assay cell growth on media lacking histidine, presumably by eliminating *HIS3* production.

Example 8 **Split-Hybrid Assay With Weakly Interacting Binding Proteins**

Protein/protein interaction was examined in the split-hybrid assay to determine utility of the system using two fusion proteins known to interact weakly. In this instance, the binding proteins were a 283 amino acid fragment of a cAMP regulatory binding protein (CREB283) fused to LexA and a fragment of the CREB binding protein consisting of the CREB binding domain (CBD) fused to VP16.

In this assay, yeast strain YI584 described above was employed and transformation carried out as previously described. In a first assay, plasmids pLexA-CREB and pVP16-CBD were transformed into the cells and cell growth was observed in the absence of histidine in the media. Expression of the fusion proteins was confirmed by Western blotting. Attempts to decrease cell growth by titration with 3AT were unsuccessful in that the concentration of 3AT required to reduce growth in cells transformed with

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pLexA-CREB and pVP16-CBD also eliminated growth in cells transformed with pLexA-CREB and the control plasmid pVP16.

In light of these results, two alternative approaches were taken in order to permit study of binding proteins wherein the interaction is relatively weak. Under the assumption that the system was failing at the level of TetR transcription, alternative approaches were taken in attempts to amplify the TetR effect on expression of *HIS3* gene. To achieve this end, assay cells were transformed with reporter constructs which encoded multiple *tet* operator sequences upstream from the *HIS3* gene. In the second approach, the *HIS3* promoter used to drive expression of the TetR gene was replaced with the stronger alcohol dehydrogenase (ADH) promoter.

In YI596 cells wherein the ADH promoter replaced the *HIS3* promoter to drive TetR expression, transformation with plasmids pLexA-CREB and pVP16-CBD showed substantially decreased growth on his⁻ media as compared to that in assay strain YI592 wherein the *HIS3* promoter was used to drive TetR expression. However, in cells transformed with plasmids pLexA-CREB 341-M1 and pVP16-CBD, no decrease in assay cell growth was detected on media lacking histidine. These results indicate that incorporation of the ADH promoter to drive TetR expression may be more useful in studies involving binding proteins that have low affinity.

When assay strains were utilized which incorporated plasmids wherein expression of the *HIS3* gene was driven by multiple copies of the *tet* operator, transformed cell lines did not grow well enough to indicate potential utility in subsequent assays.

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Example 9 General Assay Methods

A. "Fine Tuning"

In instances where either of the test fusion proteins possesses
5 intrinsic capacity for transcriptional activation, TetR will be expressed and
growth of the assay strain media lacking histidine will be depressed
proportional to the level of TetR expression. In order to restore growth of
these cells to approximately the level observed on media containing histidine,
the initially transformed assay yeast strains are grown in the presence of
10 increasing concentrations of tetracycline which binds to the TetR gene product
and prevents TetR binding to the *tet* operator. Precise titration of expressed
TetR with tetracycline, only to the point that growth of the assay strain is
restored to the level detected in the presence of histidine, permits detection of
subsequent decreased growth of the assay strain following increased TetR
15 expression resulting from interaction of the test binding proteins. The
empirically determined tetracycline concentration is therefore employed to
increase "signal-to-noise" ratios under assay conditions.

After an appropriate tetracycline concentration has been
determined for each of the candidate assay strains, the cells are transformed
20 with the second plasmid encoding the second fusion binding protein. As
before, growth of each candidate assay strain is examined on media in the
presence and absence of histidine. A desirable yeast assay strain is chosen
which shows vigorous growth in the presence of histidine and negligible
growth on media lacking histidine (indicative of the expected protein/protein
25 interaction and resultant decreased expression of *HIS3*).

In instances where binding between the two test proteins is
comparatively weak, TetR expression may not be sufficiently increased to
abolish *HIS3* expression and cells expressing the resultant low levels of *HIS3*
will still grow on media which lacks histidine. Cells which show this low
30 level of viability are grown in the presence of increasing concentrations of 3-

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aminotriazole (3AT), a competitive inhibitor in the histidine synthesis pathway, in order to reduce cell growth to negligible levels when plated on media lacking histidine. As with titration of TetR with tetracycline, addition of 3AT to the media is designed to increase the signal-to-noise ratio by providing significant changes in growth in the presence and absence of histidine in the media.

In a practical application of the methods for fine tuning, binding between CREB and the CREB binding protein (CBP) is illustrative. Growth of the yeast strain YI584 transformed with pLexA-CBD, encoding the CREB binding domain (CBD) of CBP, and pVP16-CREB or pLexA-CBD and the control plasmid pVP16 was substantially decreased and virtually indistinguishable growth rates were detected in both instances on media lacking histidine. This observation indicated that the LexA-CBD protein product possessed sufficient transactivating capacity to eliminate *HIS3* production. In order to distinguish growth differences between assay cells transformed with either pVP16 and pVP16-CREB, increasing amounts of tetracycline were added to the media lacking histidine.

In both transformants, tetracycline was able to relieve growth repression in a dose dependent manner, and at increasing concentrations of tetracycline, the difference in growth between the two colonies was increasingly magnified, with the most distinct growth difference observed following addition of tetracycline at 10 μ g/ml. Addition of tetracycline was therefore able to overcome the intrinsic transactivating capability of the LexA-CBD fusion protein.

Because the ultimate use of the split-hybrid system is for structure-function studies, mutagenesis studies, drug identification and library screens, it is important to minimize background growth that might be confused with disrupted protein-protein associations. This can be accomplished by the addition of 3AT, a competitive inhibitor of the *HIS3* gene product. For instance, in the presence of 10 μ g/ml of tetracycline, the yeast strain

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transformed with pLexA-CBD and pVP16-CREB still conferred approximately 12% growth of that observed in the presence of his⁺ media. To diminish this background, increasing concentrations of 3AT were added to the media in the presence of 10 µg/ml of tetracycline. At the 3AT concentration of 0.25 mM, the growth of the yeast strain expressing LexA-CBD and VP16-CREB was below 5%, while the growth of the control strain was still maintained at 70% of control levels. These results indicate that split-hybrid system can be modulated by 3AT in addition to tetracycline in order to effectively increase the signal-to-noise ratio.

10 B. Preparation of yeast extracts

In order to assess the utility of various plasmids to function in the split-hybrid assay, a number of control experiments can be employed which lend insight into expression of a desired protein from the transformed plasmid. For example, standard immunological methodologies, *i.e.*, immunoprecipitation, ELISA, *etc.*, can be used to determine to the extent to which a desired protein is expressed. Similarly, a variation of the gel shift assay (discussed immediately hereafter) can be used to determine both if a protein is expressed and if the expressed protein is capable of DNA binding. In each of these control assays, a yeast extract is required which can be prepared as follows.

Extracts were prepared as described by Uppaluri and Towle [*Mol. Cell. Biol.* **15**:1499-1512 (1995)] and were used for electrophoretic mobility shift assays as discussed below. The yeast cells transformed with pLexA-VP16 were grown in 100 ml of selective synthetic medium lacking uracil, tryptophan, and lysine to a density of A₆₀₀ = 1. Cells were harvested and washed with 5 ml of EB (containing 0.2 M Tris-HCl, pH 8.0, 400 mM (NH₄)₂SO₄, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, and 7 mM β-mercaptoethanol). Cells were transferred to microcentrifuge tubes and collected by centrifugation. After resuspending in 200 µl EB containing 1

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mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin, a one-half volume of glass beads was added. The suspension was frozen in a -80°C freezer for 1 hour and thawed on ice. Thawed cells were vortexed at 4°C for 20 minutes, after which an additional 100 μ l EB was
5 added, and cells were left on ice for 30 minutes. The suspension was centrifuged for 5 minutes, the supernatant was transferred to a new tube which was centrifuged for 1 hour in a microcentrifuge. The supernatant was then made to 40% with (NH₄)₂SO₄ and gently rocked for 30 minutes. After a 10
10 minute centrifugation, the pellet was resuspended in 300 μ l of 10 mM HEPES, pH 8.0, 5 mM EDTA, 7 mM β -mercaptoethanol, 1 mM PMSF, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin, and 20% glycerol. The resulting suspension was dialyzed against the same buffer, and aliquots were stored at -80°C.

C. Electrophoretic mobility shift assays

15 Electrophoretic mobility shift assays were performed as described by Shih and Towle [*J. Biol. Chem.* 267:13222-13228 (1992)]. Double-stranded *tet* operator oligonucleotides were prepared by combining equivalent amounts of complementary single-stranded DNA (SEQ ID NOS: 7 and 8) in a solution containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 50 mM NaCl₂,
20 heating the mixture to 70°C for 10 minutes, and then cooling to room temperature. The annealed oligonucleotides were labeled by filling in overhanging 5' ends using the Klenow fragment of *E. coli* DNA polymerase I with [α -³²P]dCTP. Binding reactions were carried out in 20 μ l containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol,
25 5% glycerol, and 2 mg of poly[d(I·C)]. A typical reaction contained 20,000 cpm (0.5-1 ng) of end-labeled DNA with 3-5 μ g of yeast extract. Following incubation at 22°C for 30 minutes, samples were separated on a 4.5% nondenaturing polyacrylamide gel containing 50 mM Tris, 384 mM glycine, and 2 mM EDTA, pH 8.3. For competition binding experiments, the

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conditions were exactly as above except that specific and nonspecific competitor DNAs were included in the binding mixture before the yeast extract was added. The concentration of tetracycline, a competitive inhibitor of TetR/*tet* operator binding, was 1 μ M when utilized.

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Example 10
Application of the Split-Hybrid Assay to Identify Agents
That Prevent Receptor Desensitization and Drug Tachyphylaxis

Over half of the drugs that are used clinically affect the function of seven transmembrane receptors. Although many of the characteristics of these receptors are distinct, two general features appear to be conserved. One is the ability to signal through dissociation of heterotrimeric G proteins. The second is the capacity to lose responsiveness to ligand binding in a process termed desensitization which is mediated by receptor phosphorylation and the subsequent binding of factors that recognize the phosphorylated state of the receptor which prevents continued signaling. Desensitization results in an intrinsic limitation to drug action imposed by the action of the drug itself, *i.e.*, activation of a receptor by a hormone or drug initiates mechanisms that prevent subsequent responses to repeated administration of the same agent. The coupled mechanisms of activation and deactivation together have been termed "homologous desensitization," while the inability of a drug to maintain its efficacy is known as "tachyphylaxis." Even though the mechanisms underlying homologous desensitization have been worked out in great detail over the past few years, there are currently no useful pharmacological approaches available that prevent the inactivation mechanism.

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The potential clinical utility of agents that could prevent or modulate drug desensitization is enormous. Four examples where therapy is limited by the inability of receptors to maintain responsiveness to drugs include: (i) asthma wherein desensitization of airway adrenergic receptors

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renders epinephrine treatment ineffective after a period of hours; (ii) congestive heart failure wherein desensitization of adrenergic and VIP receptors, coupled with an elevation of the β adrenergic receptor kinase (β ARK), prevents the inotropic effects of endogenous regulatory hormones; (iii) Parkinson's disease, wherein dopamine receptor desensitization limits the usefulness of agents like L-Dopa; and (iv) chronic pain wherein tolerance results from opiate receptor desensitization. Indeed, it is difficult to conceive of a pharmacological modality in use today that is not limited in its effectiveness by the phenomenon of desensitization.

The biochemical basis for G protein-coupled receptor desensitization involves three classes of proteins including arrestins, kinases and G-proteins, all of which have been cloned [Lefkowitz, *Nature Biotechnology* **14**:283-286 (1996)]. Following activation of a seven transmembrane receptor, a region is phosphorylated by one or more G protein-coupled receptor kinases (known as GRKs 1-6). For example, in the β -adrenergic receptor (β AR) and rhodopsin, the cytoplasmic tail is phosphorylated [Premont, *et al.*, *J. biol. Chem.* **269**:6832-6841 (1994); Freedman, *et al.*, *J. Biol. Chem.* **270**:17953-17961 (1995); Palczewski, *et al.*, *J. Biol. Chem.* **266**:12949-12955 (1991); Palczewski, *et al.*, *J. Biol. Chem.* **270**:15294-15298 (1995)] while in the m2 muscarinic receptor, the third cytoplasmic loop is phosphorylated [Nakata, *et al.*, *Eur. J. Biochem.* **220**:29-36 (1994)]. The best characterized members of the family of G protein receptor kinases are the β AR kinase (β ARK) and rhodopsin kinase which are both membrane-associated. While rhodopsin kinase contains an intrinsic membrane targeting signal [Inglese, *et al.*, *Nature* **359**:147-150 (1992)], β ARK appears to be targeted to the membrane by association with G protein $\beta\gamma$ subunits [Pitcher, *et al.*, *Science* **257**:1264-1267 (1992); Inglese, *et al.*, *Nature* **359**:147-150 (1992)]. Once the substrate receptor for each kinase is activated, presumably by ligand binding, the kinase associates and phosphorylates serine and threonine residues on the receptor. The phosphorylated receptor then becomes a binding target for one or more

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other proteins. In the case of β AR, for example, phosphorylation allows binding of arresting which prevents association with G proteins and promotes receptor sequestration and desensitization. Using the β AR as an exemplary desensitization model, it becomes apparent that multiple steps in the pathway appear to provide potential points of regulation each of which is amenable to the split-hybrid screen to identify molecules that can block the overall desensitization pathway. Specifically in the case of β AR, the split hybrid system can be used to identify small molecules that: (i) prevent interaction between β ARK and the G protein β subunit; (ii) inhibit β ARK activity; and (iii) disrupt the β ARK:arresting complex.

A. Plasmid Constructions

The study of G-protein receptor kinases in the split-hybrid system involves three or more recombinant proteins or two or more recombinant proteins and a recombinant peptide library. In the split-hybrid system discussed above, two yeast primary expression plasmids are employed: pBTM116 [Bartel *et al.*, *Cellular Interactions in Development: a Practical Approach*, (ed) Hartley, IRL Press, Oxford, pp. 153-179 (1993)], which encodes the LexA-fusion protein and the *TRP1* selectable marker, and pVP16 [Hollenberg *et al.*, *Mol. Cell. Biol.*, **15**:3813-3822 (1995)], which encodes the VP16-fusion protein and the *LEU2* selectable marker. In order to study interactions involving more than two recombinant proteins in the split-hybrid system, however, additional selectable markers are employed. Construction of additional yeast expression plasmids which are used to examine interactions between more than two binding proteins is discussed below.

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1. Plasmid pDRM

A DNA fragment comprising the ADH promoter and LexA sites, the TetR encoding gene, the nuclear localization signal, and the ADH terminator sequence are removed from pRS306/4xLexAop/ADH::TetR with
5 *SacI*, blunt-ended, and digested with *SaII*. The fragment is isolated and ligated into pRS303/2xtetop-LYS2 which has previously been digested with *NotI*, blunt-ended, and digested with *SaII*. The resulting plasmid, designated pDRM, is integrated into the *LYS2* locus in the yeast genome as described above, and the resulting strain designated YIDRM. Placing the repressor gene
10 and selectable marker reporter gene in the *LYS2* locus allows *URA3* to be used as a selectable marker.

2. Plasmid pRSURA3

A modified version of the pRS306 vector [Sikorski *et al.*, *Genetics*, **122**:19-27 (1989)] containing the *URA3* selectable marker gene is
15 also used to encode additional recombinant proteins in the split-hybrid system. The plasmid, pRS426, has the 2 micron origin of replication inserted into a unique *AatI* site of pRS306. Plasmid pRS426 is further modified in the following manner:

(i) The ADH promoter sequence is amplified by PCR from
20 BTM116 using primers which incorporate into the amplification product the DNA sequence encoding the SV40 large T antigen nuclear localization signal (NLS) and an initiating ATG sequence 3' to the ADH promoter. The ADH promoter/NLS/ATG sequence is inserted into the polylinker of pRS426.

(ii) The ADH terminator sequence is amplified by PCR from
25 BTM116 using primers which incorporate into the product a DNA sequence encoding an antibody tag, for example, FLAG, hemagglutinin protein (HA), or thioredoxin (Thio) (FLAG, HA, and Thio antibodies are available through Santa Cruz Biotechnology, Santa Cruz, CA) and DNA sequences encoding stop codons in all three frames to the 5' end of the ADH terminator sequence.

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The antibody tag/stop codon/ADH terminator sequence is inserted into the polylinker of pRS426.

3. Plasmid pRSADE2

5 PCR is used to engineer unique restriction sites, including for example, *Bgl*II, *Eco*47III, *Mlu*I, *Nhe*I, and *Sph*I, immediately adjacent the 5' and 3' ends of the *URA3* cassette in pRSURA3. The *URA3* cassette is digested from pRSURA3 and replaced with the *ADE2* cassette which is amplified by PCR.

4. Plasmid pBTM116/AD4

10 A fragment containing the ADH promoter, polylinker, and ADH terminator is digested from pAD4 [Young *et al.*, *Proc. Nat'l. Acad. Sci. (USA)*, 86:7989-7993 (1989)] with *Bam*HI, blunt-ended and inserted into the blunt-ended *Pvu*I site of BTM116 as described [Keegan *et al.*, *Oncogene*, 12:1537-1544 (1996)], and the resulting vector designated pBTM116/AD4.
15 PCR is also used to engineer a nuclear localization signal 3' of the ADH promoter as described above. This vector contains the *TRP1* selectable marker and can encode two recombinant proteins: (i) a LexA-fusion protein and (ii) a protein expressed from the pAD4 region of the vector.

B. β ARK and G Protein β Subunit Binding

20 In a first application of the split hybrid assay, disruption of binding between the carboxy-terminal domain of β ARK, containing the pleckstrin homology (PH) domain, and the G protein β subunit ($G\beta_2$) is examined. Previous work indicates that the PH domain of β ARK interacts directly with the $\beta\gamma$ subunits of G proteins [Pitcher, J.A., *et al. Science*
25 257:1264-1267 (1992) and Touhara, K. *et al., J.Biol. Chem.* 269:10217-10220 (1994)]. Consistent with this observation is work by Pumiglia, *et al.* [Pumiglia, K.M., *et al., J.Biol.Chem.* 270:14251-14254 (1995)] which

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indicates that $G\beta_2$ interacts with Raf1 in yeast and that the interaction is disrupted by β ARK *in vitro*.

A DNA fragment containing the carboxy-terminal 222 amino acids (residues 467 to 689) of β ARK1, which includes the PH domain, is amplified by PCR from bovine β ARK1 [Pitcher *et al.*, *Science*, 257:1264-1267 (1992)] and the gel-purified amplification product is inserted into pBTM116. The resulting plasmid is designated LexA-COOH- β ARK. A DNA fragment containing the entire coding sequence of $G\beta_2$ [Fong *et al.*, *Proc. Nat'l. Acad. Sci. (USA)*, 84:3792-3796 (1987)] is PCR amplified from pGEM-11Zf(-) $G\beta_2$ [Iniguez-Lluhi *et al.*, *JBC*, 267:23409-23417 (1992)] and the gel-purified amplification product inserted into pVP16. The resulting plasmid is designated pVP16- $G\beta_2$. PCR is used in a similar manner to clone the carboxy-terminal domain of β ARK into pVP16 and $G\beta_2$ into pBTM116.

β ARK and $G\beta_2$ binding is first examined in the two-hybrid system to determine if expression of either binding partner as a fusion protein in yeast affects protein/protein interaction. Binding of the two proteins is then examined in the split hybrid assay in order to determine if protein/protein interaction is capable of abolishing growth of the assay yeast strain. As above, addition of tetracycline and/or 3-aminotriazole required to maximize the difference in growth in the presence and absence of histidine is empirically determined.

Split-hybrid yeast strains containing β ARK and $G\beta_2$ subunits are used to screen libraries of small molecules. Several types of small molecule libraries can be examined in the split-hybrid assay, including for example, chemical libraries, libraries of products naturally produced by microorganisms, animals, plants and/or marine organisms, combinatorial, recombinatorial, peptidomimetic, multiparallel synthetic collection, protein, peptide and polypeptide libraries. A library of small peptides can be cloned into pRSURA3 as described [Yang *et al.*, *Nuc. Acids Res.*, 23:1152-1156 (1995) and Colas *et al.*, *Nature*, 380:548-550)]. Peptides corresponding to the

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carboxy-terminus of β ARK or other GRKs which have previously been shown to block calcium channel desensitization in intact neurons, presumably by blocking β ARK and $G\beta_2$ binding and subsequent trafficking of β ARK to the cellular membrane [Diverse-Pierluissi, *et al.*, *Neuron* **16**:579-585 (1996)] can be identified in such a screen. Further, it is important to show that the molecules identified through the split hybrid selection affect β ARK: $G\beta$ interaction as opposed to, for example, tetracycline analogues identified in the screen that would not be useful to specifically modulate β ARK/ $G\beta_2$ binding.

B. Identification of β ARK Inhibitors

In a second approach, agents that directly inhibit β ARK function are identified in a modification of the split-hybrid system. While identification of specific β ARK inhibitors may be difficult, preliminary data from split hybrid assays using CREB/CBP binding partners indicates that the system can be used to identify serine kinase inhibitors. The serine kinase results also suggest several approaches can be employed in attempts to overcome potential problems in identifying β ARK inhibitors.

Briefly, binding between the phosphorylated G-protein coupled receptor (P-GR) and arresting is examined first in the standard two hybrid assay, followed by identification of inhibitors of P-GR/arresting binding in the split hybrid assay. For these studies, fragments of three G protein-coupled receptors are examined: the carboxy-terminal tail of β_2 AR and the third cytoplasmic loop of the m2 muscarinic receptor. A DNA fragment containing the carboxy-terminal tail of the β_2 AR (amino acids 330 to 413) is PCR amplified [Kolbilka *et al.*, *JBC*, 262:7321-7327 (1987)] and the gel purified product inserted into pBTM116/Ad4 to produce a LexA- β_2 AR fusion gene. The resulting plasmid is designated pBTM- β_2 AR/AD4. A DNA fragment containing the third cytoplasmic loop of the human m2 muscarinic receptor (nucleotides 268-324) is amplified from pGEX-I3m2 [Haga *et al.*, *JBC*,

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269:12594-12599 (1994)] by PCR and cloned into pBTM116/Ad4 creating a LexA-m2 fusion gene. The resulting plasmid is designated pBTM-m2/AD4. The entire bovine β ARK1 coding sequence [Benovic *et al.*, *Science*, 246:235-240 (1989)] is PCR amplified and cloned into the polylinker region originating from AD4 in pBTM- β_2 AR/AD4 and pBTM-m2/AD4. The resulting plasmids are designated pBTM- β_2 AR/AD4- β ARK and pBTM-m2/AD4- β ARK, respectively. PCR is used to amplify the DNA fragment containing bovine β arresting-1 (amino acids 1 to 437) [Lohse, *et al.*, *Science*, 248:1547-1550 (1990)]. This fragment is inserted into pVP16 and is designated pVP16- β arresting-1. PCR is used to amplify the DNA fragment containing rat β arresting-2 (amino acids 1 to 428) [Attramadal, *et al.*, *JBC*, 267:17882-17890 (1992)] which is inserted into pVP16 to give plasmid pVP16- β arresting-2. A PCR strategy is also used to clone arresting into the pBTM116/AD4- β ARK plasmid and the β AR and m2 fragments into pVP16. As above, the yeast split-hybrid YIDRM strain is transformed with the P-GR-arresting along with peptide libraries (cloned into pRSURA3) or grown following transformation in the presence of combinatorial drug libraries.

Inhibitors identified in the split hybrid assay should effect disruption of protein/protein interaction either by: (i) inhibiting β ARK phosphorylation of the receptor, thus preventing recognition of the receptor by arresting, or (ii) by physical disruption of binding between the receptor and arresting. Agents that allow yeast growth for trivial reasons, *i.e.*, tetracycline analogues, can be easily identified through use of simple controls.

A first potential problem to overcome in this study is that cytoplasmic β ARK enzyme must be targeted to the substrate receptor and, once targeted, must phosphorylate the receptor at appropriate sites. In normal cells, $\beta\gamma$ association serves to target β ARK to the cell membrane; the β subunit binds to both the β ARK PH domain and the isoprenylated γ subunit in association with the membrane. One possible means to encourage the necessary specific interactions is to target the binding components in the assay

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by tagging the proteins with nuclear localization signals, *i.e.*, β ARK, the receptor cytoplasmic tail, and arresting, to the nucleus. The plasmids proposed for the study of the P-GR-arresting interaction all contain nuclear localization signal sequences adjacent to recombinant gene sequence.

5 A second problem is somewhat more difficult to approach. The current model is that receptors must be activated by ligand binding before being phosphorylated by β ARK, *i.e.*, targeting of β ARK via $\beta\gamma$ is not sufficient for receptor phosphorylation. There are two possible explanations for this requirement. The first is that phosphorylation sites on the receptor are
10 masked in the absence of ligand and ligand binding causes a conformational change which "unmasks" the phosphorylation sites. If this is the case, a fragment of the receptor containing the immediate phosphorylation site may be used as the β ARK target. However, although peptides representing portions of the β AR cytoplasmic tail can be phosphorylated by β ARK, the K_m
15 for the phosphorylation reaction is poor, suggesting that the kinase may require some other part of the receptor for binding and that the unmasking of this binding site by agonist is a critical step.

This problem is addressed in two ways. In the first, the m2 muscarinic receptor is used in place of the β AR in view of previous results
20 which indicate that the m2 protein is a good substrate for β ARK. The third cytoplasmic loop of the m2 receptor serves as both the binding site and phosphorylation site for kinase and which should allow use of a LexA/m2 receptor third cytoplasmic loop fusion gene as one component in the screening system.

25 An alternative approach is to artificially mimic the activated state of the receptor. Haga, *et al.* [*J. Biol. Chem.* **269**:12594-12599 (1994)] have shown that the activity of β ARK can be stimulated *in vitro* in the presence of mastoporan, a bee venom peptide. Mastoporan is believed to mimic the cytoplasmic face of an activated receptor and has been shown to
30 increase the affinity of β ARK for a GST-m2 receptor fusion protein by over

- 67 -

four orders of magnitude. The same effect can be seen by using peptides representing the flanking regions of the m2 third cytoplasmic loop. Thus, mastoparan should also activate β ARK in the two-hybrid yeast strains, allow phosphorylation of the receptor fusion protein, and promote interaction with
5 arresting. If mastoparan is needed, oligonucleotides containing the coding and non-coding nucleotide sequences of the 14-mer peptide (INLKALAALAKKIL-NH₂, SEQ ID NO: 43) are annealed and ligated into prSADE2. The yeast split-hybrid strain YIDRM is transformed with pBTM- β AR (or m2)/AD4- β ARK, pVP16-arresting, prSADE2-mastoparan, and a pRSURA3-peptide
10 library or combinatorial drug library.

Numerous modifications and variations in the invention as set forth in the above illustrative examples are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Hoekstra, Merl F.
- (ii) TITLE OF INVENTION: Methods to Identify Compounds For Disrupting Protein/Protein Interactions
- (iii) NUMBER OF SEQUENCES: 43
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
 - (B) STREET: 6300 Sears Tower, 233 South Wacker Drive
 - (C) CITY: Chicago
 - (D) STATE: Illinois
 - (E) COUNTRY: United States of America
 - (F) ZIP: 60606-6402
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME:
 - (B) REGISTRATION NUMBER:
 - (C) REFERENCE/DOCKET NUMBER: 27866/33424
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 312/474-6300
 - (B) TELEFAX: 312/474-0448
 - (C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTGGTGAGCG CTAGGAGTCA CTGCCAG

27

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TATACTCTAT CAATGATAGA GTAATTCATT ATGTGATAAT GCC

43

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTACTCTAT CATTGATAGA GTATATAAAG TAATGTGATT TC

42

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATTCTGCTA GCCTCTGCAA AGC

23

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCACGCGTC GAAGAAATCA CATTACTTTA TATA

34

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
CGCACGCGTA TACTAAAAAA TGAGCAGGCA AG 32

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
CGCGTACTCT ATCATTGATA GAGTA 25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
ATGAGATAGT AACTATCTCA TGCGC 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
CGCGTACTCT ATCATTGATA GAGTCTAGAC TCTATCAATG ATAGAGTA 48

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
GCGACGCGTG CATGCCGTCT TCAAGAATTC CTCGAG 36

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(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCGACGCGTG CATGCCCACC GTACACGCCT ACTCGA

36

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATGGCATGC AAAAAAAAAAG AGTCATCCGC TAGG

34

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CATGGCATGC TTAGCGATTG GCATTATCAC AT

32

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TAATACGACT CACTATATAG GG

22

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTAGACTTT GCCTTCGTTT ATC 23

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGAAGGCAAA GATGTCTAGA TTAGATAAAA G 31

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGCGGATCCG CTTTCTCTTC TTTTTTGGAG ACCCACTTTC ACATTTAAG 49

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AATTGCTCGA GTACTGTATG TACATACAGT AG 32

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AATTCTACTG TATGTACATA CAGTACTCGA GC 32

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(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCGGAATTCT CGAGACATAT CCATATCTAA TC

32

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCGGAATTCA CTAATCGCAT TATCATC

27

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CATGCCATGG CCATGTCTAG ATTAGATAAA AG

32

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCGAATTCGC CAGGGCAACA GAATGCCACT

30

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGGGATCCTG GCTGGTTACC CAGGATGCCT TG

32

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGCGGATCCG GATGACCATG GACTCTGGAG
30

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CGCGGATCCT TAATCTGACT TGTGGCAGTA
30

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CGCGGATCCC CATGACCATG GAATCTGGAG CC
32

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGCGGATCCG TGCTGCTTCT TCAGCAGGCT G
31

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATGGTACCAG CGGCCGCTAG TCGTTTTACA ACGTCGTGAC

40

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATGGTACCGC GGCCGCTTAT TTTTGACACC AGACCAAC
38

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CGGAGATCTA AAGAGACTTT TCTCCGGAAC TCAG
34

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGGAGATCTT TACAGGAAGA CTGAACTGT
29

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(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCACCGCGGC AGTGCCAACC CCGATTAC

29

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CATCCGCGGT GGTGATGGCA GGGGCTGA

28

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGCTATCGAT ACGGCCCCC CGACCGAT

28

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GCGTATCGAT CTACCCACCG TACTCGTC

28

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs

- 77 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCTACTCTTA GGCCCGGGTC TTTTAAATGT ATCC
34

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GGAATCACTA CAGGGATG
18

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1485 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

ATGGACTTAA GAGTAGGAAG GAAATTTCTGT ATTGGCAGGA AGATTGGGAG TGGTTCCTTT	60
GGTGACATTT ACCACGGCAC GAACTTAATT AGTGGTGAAG AAGTAGCCAT CAAGCTGGAA	120
TCGATCAGGT CCAGACATCC TCAATTGGAC TATGAGTCCC GCGTCTACAG ATACTTAAGC	180
GGTGGTGTGG GAATCCCGTT CATCAGATGG TTTGGCAGAG AGGGTGAATA TAATGCTATG	240
GTCATCGATC TTCTAGGCC ATCTTTGGAA GATTTATTCA ACTACTGTCA CAGAAGGTTC	300
TCCTTTAAGA CGGTATCAT GCTGGCTTTG CAAATGTTTT GCCGTATTCA GTATATACAT	360
GGAAGGTCGT TCATTCATAG AGATATCAAA CCAGACAAC TTTAATGGG GGTAGGACGC	420
CGTGGTAGCA CCGTTCATGT TATTGATTTT GGTCTATCAA AGAAATACCG AGATTTCAAC	480
ACACATCGTC ATATTCCTTA CAGGGAGAAC AAGTCCTTGA CAGGTACAGC TCGTTATGCA	540
AGTGTCAATA CGCATCTTGG AATAGAGCAA AGTAGAAGAG ATGACTTAGA ATCACTAGGT	600
TATGTCTTGA TCTATTTTGT TAAGGGTTCT TTGCCATGGC AGGGTTTGAA AGCAACCACC	660
AAGAAACAAA AGTATGATCG TATCATGGAA AAGAAATTAA ACGTTAGCGT GGAAACTCTA	720
TGTTTCAGGT TACCATTAGA GTTTCAAGAA TATATGGCTT ACTGTAAGAA TTTGAAATTC	780
GATGAGAAGC CAGATTATTT GTTCTTGGCA AGGCTGTTTA AAGATCTGAG TATTAAACTA	840
GAGTATCACA ACGACCACTT GTTCGATTGG ACAATGTTGC GTTACACAAA GGCGATGGTG	900
GAGAAGCAAA GGGACCTCCT CATCGAAAAA GGTGATTTGA ACGCAAATAG CAATGCAGCA	960
AGTGCAAGTA ACAGCACAGA CAACAAGTCT GAACTTTCA ACAAGATTAA ACTGTTAGCC	1020
ATGAAGAAAT TCCCCACCCA TTTCCACTAT TACAAGAATG AAGACAAACA TAATCCTTCA	1080
CCAGAAGAGA TCAACAACA AACTATCTTG AATAATAATG CAGCCTCTTC TTTACCAGAG	1140
GAATTATTGA ACGCACTAGA TAAAGGTATG GAAAACCTGA GACAACAGCA GCCGCAGCAG	1200
CAGGTCCAAA GTTCGCAGCC ACAACCACAG CCCCAACAGC TACAGCAGCA ACCAAATGGC	1260
CAAAGACCAA ATTATTATCC TGAACCGTTA CTACAGCAGC AACAAAGAGA TTCTCAGGAG	1320
CAACAGCAGC AAGTTCCGAT GGCTACAACC AGGGCTACTC AGTATCCCCC ACAAATAAAC	1380
AGCAATAATT TTAATACTAA TCAAGCATCT GTACCTCCAC AAATGAGATC TAATCCACAA	1440
CAGCCGCCTC AAGATAAACC AGCTGGCCAG TCAATTTGGT TGTA	1485

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2625 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 796..2580

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CATTTTCTTA ATTCTTTTAT GTGCTTTTAC TACTTTGTTT AGTTCAAAAC AATAGTCGTT	60
ATTCTTAGGT ACTATAGCAT AAGACAAGAA AAGAAAAATA AGGGACAAAT AACATTAGCA	120
GAAGTACGGT ATATTTTACT GTTACTTATA TACTTTCAAG AAGATGAGTT AAATCGGTAG	180
CCAGTGTAGA AAAATAATAA TAAGGGTCAT CGATCCTTCG CATTTTATTA TCCAATTAAA	240
GATACGAATC ACGGCAAAC ATATTCAAAG CTCATAGATA ATCGTCGTAA GGCTGACACT	300
GCAGAAGAAA AGTCATAATT TGAATACTAG CCGGTATGAA ACTGTGATTG ATTAACCTGG	360
GGTTACCTAA AGAGAACATA AGTAATACTC ATGACAGAAT CAAAACACAA TACAAAATTT	420
ATCCGAACCT CGGCCCGACT GCGGCTCGCC GGGAAAGGGG ACAACCGCTT CTATCCGTCG	480
ACTAACTTCA TCGGCCCAAT GGAAGCTATG ATATGGGGAT TTCCATTGAG CCGATAGCAA	540
TGTAGGGTAA TACTGTTGCG TATATAGTGA TAGTTATTGA ATTTTATTAC CCTGCGGGAA	600
TATTGAGACA TCACTAAGCA CGAATTTTAC GTCTGAGGAA AGTTGAATGA TGGCCAAATA	660
ACCAGGAAAA ACAATATTG AATCCTTGTG AAGGATTCCA CAGTTGTTTA ATCCTCCTTA	720
AGCTCACTTA GTATCAATTG TCTAAATAAT ATTGCTTTGA ATCTGAAAAA AATAAAAGTA	780
CCTTCGCATT AGACA ATG TCA CTG CCG CTA CGA CAC GCA TTG GAG AAC GTT	831
Met Ser Leu Pro Leu Arg His Ala Leu Glu Asn Val	
1 5 10	
ACT TCT GTT GAT AGA ATT TTA GAG GAC TTA TTA GTA CGT TTT ATT ATA	879
Thr Ser Val Asp Arg Ile Leu Glu Asp Leu Leu Val Arg Phe Ile Ile	
15 20 25	
AAT TGT CCG AAT GAA GAT TTA TCG AGT GTC GAG AGA GAG TTA TTT CAT	927
Asn Cys Pro Asn Glu Asp Leu Ser Ser Val Glu Arg Glu Leu Phe His	
30 35 40	
TTT GAA GAA GCC TCA TGG TTT TAC ACG GAT TTC ATC AAA TTG ATG AAT	975
Phe Glu Glu Ala Ser Trp Phe Tyr Thr Asp Phe Ile Lys Leu Met Asn	
45 50 55 60	
CCA ACT TTA CCC TCC CTA AAG ATT AAA TCA TTT GCT CAA TTG ATC ATA	1023
Pro Thr Leu Pro Ser Leu Lys Ile Lys Ser Phe Ala Gln Leu Ile Ile	
65 70 75	
AAA CTA TGT CCT CTG GTT TGG AAA TGG GAC ATA AGA GTG GAT GAG GCA	1071
Lys Leu Cys Pro Leu Val Trp Lys Trp Asp Ile Arg Val Asp Glu Ala	
80 85 90	
CTC CAG CAA TTC TCC AAG TAT AAG AAA AGT ATA CCG GTG AGG GGC GCT	1119
Leu Gln Gln Phe Ser Lys Tyr Lys Lys Ser Ile Pro Val Arg Gly Ala	
95 100 105	
GCC ATA TTT AAC GAG AAC CTG AGT AAA ATT TTA TTG GTA CAG GGT ACT	1167
Ala Ile Phe Asn Glu Asn Leu Ser Lys Ile Leu Leu Val Gln Gly Thr	

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110	115	120	
GAA TCG GAT TCT TTG TCA TTC CCA AGG GGG AAG ATA TCT AAA GAT GAA Glu Ser Asp Ser Leu Ser Phe Pro Arg Gly Lys Ile Ser Lys Asp Glu 125 130 135 140			1215
AAT GAC ATA GAT TGT TGC ATT AGA GAA GTG AAA GAA GAA ATT GGT TTC Asn Asp Ile Asp Cys Cys Ile Arg Glu Val Lys Glu Glu Ile Gly Phe 145 150 155			1263
GAT TTG ACG GAC TAT ATT GAC GAC AAC CAA TTC ATT GAA AGA AAT ATT Asp Leu Thr Asp Tyr Ile Asp Asp Asn Gln Phe Ile Glu Arg Asn Ile 160 165 170			1311
CAA GGT AAA AAT TAC AAA ATA TTT TTG ATA TCT GGT GTT TCA GAA GTC Gln Gly Lys Asn Tyr Lys Ile Phe Leu Ile Ser Gly Val Ser Glu Val 175 180 185			1359
TTC AAT TTT AAA CCT CAA GTT AGA AAT GAA ATT GAT AAG ATA GAA TGG Phe Asn Phe Lys Pro Gln Val Arg Asn Glu Ile Asp Lys Ile Glu Trp 190 195 200			1407
TTC GAT TTT AAG AAA ATT TCT AAA ACA ATG TAC AAA TCA AAT ATC AAG Phe Asp Phe Lys Lys Ile Ser Lys Thr Met Tyr Lys Ser Asn Ile Lys 205 210 215 220			1455
TAT TAT CTG ATT AAT TCC ATG ATG AGA CCC TTA TCA ATG TGG TTA AGG Tyr Tyr Leu Ile Asn Ser Met Met Arg Pro Leu Ser Met Trp Leu Arg 225 230 235			1503
CAT CAG AGG CAA ATA AAA AAT GAA GAT CAA TTG AAA TCC TAT GCG GAA His Gln Arg Gln Ile Lys Asn Glu Asp Gln Leu Lys Ser Tyr Ala Glu 240 245 250			1551
GAA CAA TTG AAA TTG TTG TTG GGT ATC ACT AAG GAG GAG CAG ATT GAT Glu Gln Leu Lys Leu Leu Leu Gly Ile Thr Lys Glu Glu Gln Ile Asp 255 260 265			1599
CCC GGT AGA GAG TTG CTG AAT ATG TTA CAT ACT GCA GTG CAA GCT AAC Pro Gly Arg Glu Leu Leu Asn Met Leu His Thr Ala Val Gln Ala Asn 270 275 280			1647
AGT AAT AAT AAT GCG GTC TCC AAC GGA CAG GTA CCC TCG AGC CAA GAG Ser Asn Asn Asn Ala Val Ser Asn Gly Gln Val Pro Ser Ser Gln Glu 285 290 295 300			1695
CTT CAG CAT TTG AAA GAG CAA TCA GGA GAA CAC AAC CAA CAG AAG GAT Leu Gln His Leu Lys Glu Gln Ser Gly Glu His Asn Gln Gln Lys Asp 305 310 315			1743
CAG CAG TCA TCG TTT TCT TCT CAA CAA CAA CCT TCA ATA TTT CCA TCT Gln Gln Ser Ser Phe Ser Ser Gln Gln Pro Ser Ile Phe Pro Ser 320 325 330			1791
CTT TCT GAA CCG TTT GCT AAC AAT AAG AAT GTT ATA CCA CCT ACT ATG Leu Ser Glu Pro Phe Ala Asn Asn Lys Asn Val Ile Pro Pro Thr Met 335 340 345			1839
CCA ATG GCT AAC GTA TTC ATG TCA AAT CCT CAA TTG TTT GCG ACA ATG Pro Met Ala Asn Val Phe Met Ser Asn Pro Gln Leu Phe Ala Thr Met 350 355 360			1887
AAT GGC CAG CCT TTT GCA CCT TTC CCA TTT ATG TTA CCA TTA ACT AAC			1935

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365	Asn	Gly	Gln	Pro	Phe	Ala	Pro	Phe	Pro	Phe	Met	Leu	Pro	Leu	Thr	Asn	380	
AAT	AGT	AAT	AGC	GCT	AAC	CCT	ATT	CCA	ACT	CCG	GTC	CCC	CCT	AAT	TTT		1983	
Asn	Ser	Asn	Ser	Ala	Asn	Pro	Ile	Pro	Thr	Pro	Val	Pro	Pro	Asn	Phe			
				385					390					395				
AAT	GCT	CCT	CCG	AAT	CCG	ATG	GCT	TTT	GGT	GTT	CCA	AAC	ATG	CAT	AAC		2031	
Asn	Ala	Pro	Pro	Asn	Pro	Met	Ala	Phe	Gly	Val	Pro	Asn	Met	His	Asn			
			400					405					410					
CTT	TCT	GGA	CCA	GCA	GTA	TCT	CAA	CCG	TTT	TCC	TTG	CCT	CCT	GCT	CCT		2079	
Leu	Ser	Gly	Pro	Ala	Val	Ser	Gln	Pro	Phe	Ser	Leu	Pro	Pro	Ala	Pro			
		415					420					425						
TTA	CCG	AGG	GAC	TCT	GGT	TAC	AGC	AGC	TCC	TCC	CCT	GGG	CAG	TTG	TTA		2127	
Leu	Pro	Arg	Asp	Ser	Gly	Tyr	Ser	Ser	Ser	Ser	Pro	Gly	Gln	Leu	Leu			
	430					435					440							
GAT	ATA	CTA	AAT	TCG	AAA	AAG	CCT	GAC	AGC	AAC	GTG	CAA	TCA	AGC	AAA		2175	
Asp	Ile	Leu	Asn	Ser	Lys	Lys	Pro	Asp	Ser	Asn	Val	Gln	Ser	Ser	Lys			
445					450					455					460			
AAG	CCA	AAG	CTT	AAA	ATC	TTA	CAG	AGA	GGA	ACG	GAC	TTG	AAT	TCA	CTC		2223	
Lys	Pro	Lys	Leu	Lys	Ile	Leu	Gln	Arg	Gly	Thr	Asp	Leu	Asn	Ser	Leu			
				465					470					475				
AAG	CAA	AAC	AAT	AAT	GAT	GAA	ACT	GCT	CAT	TCA	AAC	TCT	CAA	GCT	TTG		2271	
Lys	Gln	Asn	Asn	Asn	Asp	Glu	Thr	Ala	His	Ser	Asn	Ser	Gln	Ala	Leu			
			480					485					490					
CTA	GAT	TTG	TTG	AAA	AAA	CCA	ACA	TCA	TCG	CAG	AAG	ATA	CAC	GCT	TCC		2319	
Leu	Asp	Leu	Leu	Lys	Lys	Pro	Thr	Ser	Ser	Gln	Lys	Ile	His	Ala	Ser			
		495					500					505						
AAA	CCA	GAT	ACT	TCC	TTT	TTA	CCA	AAT	GAC	TCC	GTA	TCT	GGT	ATA	CAA		2367	
Lys	Pro	Asp	Thr	Ser	Phe	Leu	Pro	Asn	Asp	Ser	Val	Ser	Gly	Ile	Gln			
	510					515					520							
GAT	GCA	GAA	TAT	GAA	GAT	TTC	GAG	AGT	AGT	TCA	GAT	GAA	GAG	GTG	GAG		2415	
Asp	Ala	Glu	Tyr	Glu	Asp	Phe	Glu	Ser	Ser	Ser	Asp	Glu	Glu	Val	Glu			
525					530					535					540			
ACA	GCT	AGA	GAT	GAA	AGA	AAT	TCA	TTG	AAT	GTA	GAT	ATT	GGG	GTG	AAC		2463	
Thr	Ala	Arg	Asp	Glu	Arg	Asn	Ser	Leu	Asn	Val	Asp	Ile	Gly	Val	Asn			
				545					550				555					
GTT	ATG	CCA	AGC	GAA	AAA	GAC	AGC	CGA	AGA	AGT	CAA	AAG	GAA	AAA	CCA		2511	
Val	Met	Pro	Ser	Glu	Lys	Asp	Ser	Arg	Arg	Ser	Gln	Lys	Glu	Lys	Pro			
			560					565					570					
AGG	AAC	GAC	GCA	AGC	AAA	ACA	AAC	TTG	AAC									

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(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6854 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2050..4053

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AGCTTCTCCC	TTTTCCTTCA	GTGCTGCTAC	TCTCTGCTCT	CCACTTAAGT	GTTACAATTA	60
ATTTGCAGCT	AGTTTGCAGT	TCGTACAACC	TCGCCTATTC	TTGTAACGAA	GAAGAACGTA	120
TTTATAATAT	TGGGCTGTAA	TGTGTTGAGT	TTAGTAATAG	ATAAAGTAGG	ACAGAGTTCT	180
GTCTTTGTTT	ATCTATGGGG	TTCAGAGTGA	TAAGGGGCAG	GATAAGGAAG	TTAAAAAAAA	240
AAAGGTTACG	TTATATAACG	AAAGAAAAGA	AACGAGCGAA	GTGCCAACTA	TAGCCCAATA	300
TCAAGAATGC	AAGTCAGCAA	AGTACAGTAA	TCGTATGAAG	ATACGCGATG	CGTAATATCC	360
CTCAAGGGCT	CCGGATCAGA	AAAGCTAAGG	GAAGATCCTT	ACATTACACG	GCGTGCGACA	420
GACTCGAACC	ACAGCTAACT	TCTCGTGAAA	AGATGGCTTC	AACTTCGCTC	TTGCAATAAC	480
TTTGAAACAC	ACGAACAAAG	GTTTATTGCG	CTTGATTAACT	GTTGGAAGTA	TATGATACTA	540
ATACTACTTT	GTTCTCTAAG	TCATCGCTAT	ATGTTTATCT	CGAGGAAAAG	GTGCACGGCG	600
GTACACAATT	ACTTCGCCGT	TTCGGGTAAA	ACAAGTGTTA	CATTTATAAT	ATATATGTAT	660
ATATGTATGT	GCGCGTAAGT	ATATGCCGTT	CATAACAAAT	CATCTTCTTG	TTGCTGGATG	720
GACTCCTTAA	TTTTATTCAA	AATGGTAATT	TTCCATTTAT	CTAGTCTCAT	AAAATTGTCA	780
AACTCCTTAC	AGTGTTGCTC	TAGCTGCTCG	CTATCACCTT	CATTAACAGC	ATCGATTAAA	840
CTTTTCAAGA	AATTTGACTC	CCTTGAATCC	GCAAAATTCG	GATCTTCACT	TTGACCCTCT	900
TGTAAAGTTC	TTGCAGCAGC	GACTGCATCA	GTAGCAGCTA	GCTGACAAAG	CCCTTTTTTT	960
AGGAAGTAAT	CCTTCAAAC	CCATTGGCTC	AATCTATTGC	CCATGCTGCT	CTTGATCAAC	1020
TTCGAATATA	TATCACTTGC	TTCAATATAT	TGACCGTCAA	GAGCCTTTAG	ATCTGCGCAT	1080
TTGATAAAAC	ACTTATTGCA	TAATGCTACC	GACTGGTCTT	GGGCATACCA	CTCACCAGCG	1140
AGCTCATAGC	AATCTATAGC	TTTTGCATAG	TCATGCAAAT	CATTTTCTAG	AATTTCTCCA	1200
AGCTCAAAC	TGAAATTAGC	ACCTCTCCGG	AACTGCCCCC	TATGAGTAAA	AATTTGAATA	1260
GCATTTTCTA	ATGAATCCAC	GGCGTTCACA	GAGTTTCCAC	CGCTTTTAAA	GCATTTATAA	1320
GCCTCTACGT	AGGTATTTCC	TGCTTCGTCT	TCATTACCAG	CCTTTTCTTG	ATAGTCAGCA	1380

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GCTTTCAAAA	ACGAGTCTCC	TGCCAAGTTT	AACTCTTTTC	TTAGACGGTA	AATGGTGGCT	1440
GCTTGGACAC	AAAGATCAGC	AGCCTCCTCA	AACTTGATATG	AATCAGAACC	GCTAAACAAT	1500
TTCATGAAAC	CCGATGAAGG	AACACCCTTC	TTCTCAGCCT	TAACACAACG	GGAAATATCA	1560
ATTCCCGTAT	TTCAATGTTA	GTAATTTGCC	TTCGTAAATT	ACGGAATCAC	ATAGCTTTCA	1620
TTTTGTTCCT	TTGATATATT	TCCCTACTAC	ATACTCTTTT	CAATAACTCT	ACAGGGTCTG	1680
ACATTTTTTAA	CTTTCAGGTT	AATGATGGTG	TTCTTACTAT	ATTCTCGAGT	CGTACAGAAG	1740
TTAGTTCAGA	TAAACTGCTT	CGGTGCTGCC	CACTTCTTAT	CATTACTTCA	ACTTTACCTT	1800
CCCTATACCT	GTGTGTCCTT	ATTAATTCAA	GTTAATCCGA	GGTAATAGAT	TAGGGTAACC	1860
TTCAATGATG	TCACGAAACA	CGGATGCTGC	AACTTTGCGA	TTTTTTCCTG	GAAAAGAATA	1920
ACAATTAAAG	GCAGCCTTTC	AGCTGAGATT	ACCAGCAGGT	CTTTGGAGAT	TAGCGCAAGA	1980
AGAAGTGTGA	TATAGTACTC	ATAGAGGCAG	GCTACAGACT	AGGGAAAGCG	TGTTCAACAA	2040
CAATAAGAA	ATG GAG ACC AGT TCT TTT GAG AAT GCT CCT CCT GCA GCC					2088
	Met Glu Thr Ser Ser Phe Glu Asn Ala Pro Pro Ala Ala					
	1 5 10					
ATC AAT GAT GCT CAG GAT AAT AAT ATA AAT ACG GAG ACT AAT GAC CAG						2136
Ile Asn Asp Ala Gln Asp Asn Asn Ile Asn Thr Glu Thr Asn Asp Gln						
15 20 25						
GAA ACA AAT CAG CAA TCT ATC GAA ACT AGA GAT GCA ATT GAC AAA GAA						2184
Glu Thr Asn Gln Gln Ser Ile Glu Thr Arg Asp Ala Ile Asp Lys Glu						
30 35 40 45						
AAC GGT GTG CAA ACG GAA ACT GGT GAG AAC TCT GCA AAA AAT GCC GAA						2232
Asn Gly Val Gln Thr Glu Thr Gly Glu Asn Ser Ala Lys Asn Ala Glu						
50 55 60						
CAA AAC GTT TCT TCT ACA AAT TTG AAT AAT GCC CCC ACC AAT GGT GCT						2280
Gln Asn Val Ser Ser Thr Asn Leu Asn Asn Ala Pro Thr Asn Gly Ala						
65 70 75						
TTG GAC GAT GAT GTT ATC CCA AAT GCT ATT GTT ATT AAA AAC ATT CCG						2328
Leu Asp Asp Asp Val Ile Pro Asn Ala Ile Val Ile Lys Asn Ile Pro						
80 85 90						
TTT GCT ATT AAA AAA GAG CAA TTG TTA GAC ATT ATT GAA GAA ATG GAT						2376
Phe Ala Ile Lys Lys Glu Gln Leu Leu Asp Ile Ile Glu Glu Met Asp						
95 100 105						
CTT CCC CTT CCT TAT GCC TTC AAT TAC CAC TTT GAT AAC GGT ATT TTC						2424
Leu Pro Leu Pro Tyr Ala Phe Asn Tyr His Phe Asp Asn Gly Ile Phe						
110 115 120 125						
AGA GGA CTA GCC TTT GCG AAT TTC ACC ACT CCT GAA GAA ACT ACT CAA						2472
Arg Gly Leu Ala Phe Ala Asn Phe Thr Thr Pro Glu Glu Thr Thr Gln						
130 135 140						
GTG ATA ACT TCT TTG AAT GGA AAG GAA ATC AGC GGG AGG AAA TTG AAA						2520
Val Ile Thr Ser Leu Asn Gly Lys Glu Ile Ser Gly Arg Lys Leu Lys						
145 150 155						

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GTG	GAA	TAT	AAA	AAA	ATG	CTT	CCC	CAA	GCT	GAA	AGA	GAA	AGA	ATC	GAG	2568
Val	Glu	Tyr	Lys	Lys	Met	Leu	Pro	Gln	Ala	Glu	Arg	Glu	Arg	Ile	Glu	
		160					165					170				
AGG	GAG	AAG	AGA	GAG	AAA	AGA	GGA	CAA	TTA	GAA	GAA	CAA	CAC	AGA	TCG	2616
Arg	Glu	Lys	Arg	Glu	Lys	Arg	Gly	Gln	Leu	Glu	Glu	Gln	His	Arg	Ser	
	175					180					185					
TCA	TCT	AAT	CTT	TCT	TTG	GAT	TCT	TTA	TCT	AAA	ATG	AGT	GGA	AGC	GGA	2664
Ser	Ser	Asn	Leu	Ser	Leu	Asp	Ser	Leu	Ser	Lys	Met	Ser	Gly	Ser	Gly	
190					195					200					205	
AAC	AAT	AAT	ACT	TCT	AAC	AAT	CAA	TTA	TTC	TCG	ACT	CTA	ATG	AAC	GGC	2712
Asn	Asn	Asn	Thr	Ser	Asn	Asn	Gln	Leu	Phe	Ser	Thr	Leu	Met	Asn	Gly	
				210					215					220		
ATT	AAT	GCT	AAT	AGC	ATG	ATG	AAC	AGT	CCA	ATG	AAT	AAT	ACC	ATT	AAC	2760
Ile	Asn	Ala	Asn	Ser	Met	Met	Asn	Ser	Pro	Met	Asn	Asn	Thr	Ile	Asn	
			225					230					235			
AAT	AAC	AGT	TCT	AAT	AAC	AAC	AAT	AGT	GGT	AAC	ATC	ATT	CTG	AAC	CAA	2808
Asn	Asn	Ser	Ser	Asn	Asn	Asn	Asn	Ser	Gly	Asn	Ile	Ile	Leu	Asn	Gln	
		240					245					250				
CCT	TCA	CTT	TCT	GCC	CAA	CAT	ACT	TCT	TCA	TCG	TTG	TAC	CAA	ACA	AAC	2856
Pro	Ser	Leu	Ser	Ala	Gln	His	Thr	Ser	Ser	Ser	Leu	Tyr	Gln	Thr	Asn	
	255					260					265					
GTT	AAT	AAT	CAA	GCC	CAG	ATG	TCC	ACT	GAG	AGA	TTT	TAT	GCG	CCT	TTA	2904
Val	Asn	Asn	Gln	Ala	Gln	Met	Ser	Thr	Glu	Arg	Phe	Tyr	Ala	Pro	Leu	
270					275					280					285	
CCA	TCA	ACT	TCC	ACT	TTG	CCT	CTC	CCA	CCC	CAA	CAA	CTG	GAC	TTC	AAT	2952
Pro	Ser	Thr	Ser	Thr	Leu	Pro	Leu	Pro	Pro	Gln	Gln	Leu	Asp	Phe	Asn	
				290				295						300		
GAC	CCT	GAC	ACT	TTG	GAA	ATT	TAT	TCC	CAA	TTA	TTG	TTA	TTT	AAG	GAT	3000
Asp	Pro	Asp	Thr	Leu	Glu	Ile	Tyr	Ser	Gln	Leu	Leu	Leu	Phe	Lys	Asp	
			305					310					315			
AGA	GAA	AAG	TAT	TAT	TAC	GAG	TTG	GCT	TAT	CCC	ATG	GGT	ATA	TCC	GCT	3048
Arg	Glu	Lys	Tyr	Tyr	Tyr	Glu	Leu	Ala	Tyr	Pro	Met	Gly	Ile	Ser	Ala	
		320				325						330				
TCC	CAC	AAG	AGA	ATT	ATC	AAT	GTT	TTG	TGC	TCG	TAC	TTA	GGG	CTA	GTA	3096
Ser	His	Lys	Arg	Ile	Ile	Asn	Val	Leu	Cys	Ser	Tyr	Leu	Gly	Leu	Val	
	335				340						345					
GAA	GTA	TAT	GAT	CCA	AGA	TTT	ATT	ATT	ATC	AGA	AGA	AAG	ATT	CTG	GAT	3144
Glu	Val	Tyr	Asp	Pro	Arg	Phe	Ile	Ile	Ile	Arg	Arg	Lys	Ile	Leu	Asp	
350					355					360					365	
CAT	GCT	AAT	TTA	CAA	TCT	CAT	TTG	CAA	CAA	CAA	GGT	CAA	ATG	ACA	TCT	3192
His	Ala	Asn	Leu	Gln	Ser	His	Leu	Gln	Gln	Gln	Gly	Gln	Met	Thr	Ser	
				370				375						380		
GCT	CAT	CCT	TTG	CAG	CCA	AAC	TCC	ACT	GGC	GGC	TCC	ATG	AAT	AGG	TCA	3240
Ala	His	Pro	Leu	Gln	Pro	Asn	Ser	Thr	Gly	Gly	Ser	Met	Asn	Arg	Ser	
			385					390					395			
CAA	TCT	TAT	ACA	AGT	TTG	TTA	CAG	GCC	CAT	GCA	GCA	GCT	GCA	GCG	AAT	3288
Gln	Ser	Tyr	Thr	Ser	Leu	Leu	Gln	Ala	His	Ala	Ala	Ala	Ala	Ala	Asn	
		400					405					410				

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AGT Ser 415	ATT Ile	AGC Ser	AAT Asn	CAG Gln	GCC Ala	GTT Val 420	AAC Asn	AAT Asn	TCT Ser	TCC Ser	AAC Asn 425	AGC Ser	AAT Asn	ACT Thr	ATT Ile	3336
AAC Asn 430	AGT Ser	AAT Asn	AAC Asn	GGT Gly	AAC Asn 435	GGT Gly	AAC Asn	AAT Asn	GTC Val	ATC Ile 440	ATT Ile	AAT Asn	AAC Asn	AAT Asn	AGC Ser 445	3384
GCC Ala	AGC Ser	TCA Ser	ACA Thr	CCA Pro 450	AAA Lys	ATT Ile	TCT Ser	TCA Ser	CAG Gln 455	GGA Gly	CAA Gln	TTC Phe	TCC Ser	ATG Met 460	CAA Gln	3432
CCA Pro	ACA Thr	CTA Leu	ACC Thr 465	TCA Ser	CCT Pro	AAA Lys	ATG Met	AAC Asn 470	ATA Ile	CAC His	CAT His	AGT Ser	TCT Ser 475	CAA Gln	TAC Tyr	3480
AAT Asn	TCC Ser	GCA Ala 480	GAC Asp	CAA Gln	CCG Pro	CAA Gln 485	CAA Gln	CCT Pro	CAA Gln	CCA Pro	CAA Gln	ACA Thr 490	CAG Gln	CAA Gln	AAT Asn	3528
GTT Val 495	CAG Gln	TCA Ser	GCT Ala	GCG Ala	CAA Gln	CAA Gln 500	CAA Gln	CAA Gln	TCT Ser	TTT Phe	TTA Leu 505	AGA Arg	CAA Gln	CAA Gln	GCT Ala	3576
ACT Thr 510	TTA Leu	ACA Thr	CCA Pro	TCC Ser	TCA Ser 515	AGA Arg	ATT Ile	CCA Pro	TCC Ser	GGT Gly 520	TAT Tyr	TCT Ser	GCC Ala	AAC Asn	CAT His 525	3624
TAT Tyr	CAA Gln	ATC Ile	AAT Asn	TCC Ser 530	GTT Val	AAT Asn	CCC Pro	TTA Leu	CTG Leu 535	AGA Arg	AAT Asn	TCT Ser	CAA Gln	ATT Ile 540	TCA Ser	3672
CCT Pro	CCA Pro	AAT Asn	TCA Ser 545	CAA Gln	ATC Ile	CCA Pro	ATC Ile	AAC Asn 550	AGC Ser	CAA Gln	ACC Thr	CTA Leu	TCC Ser 555	CAA Gln	GCG Ala	3720
CAA Gln	CCA Pro	CCA Pro	GCA Ala 560	CAG Gln	TCC Ser	CAA Gln	ACT Thr 565	CAA Gln	CAA Gln	CGG Arg	GTA Val 570	CCA Pro	GTG Val	GCA Ala	TAC Tyr	3768
CAA Gln 575	AAT Asn	GCT Ala	TCA Ser	TTG Leu	TCT Ser	TCC Ser 580	CAG Gln	CAG Gln	TTG Leu	TAC Tyr	AAC Asn 585	CTT Leu	AAC Asn	GGC Gly	CCA Pro	3816
TCT Ser 590	TCA Ser	GCA Ala	AAC Asn	TCA Ser	CAG Gln 595	TCC Ser	CAA Gln	CTG Leu	CTT Leu	CCA Pro 600	CAG Gln	CAC His	ACA Thr	AAT Asn	GGC Gly 605	3864
TCA Ser	GTA Val	CAT His	TCT Ser	AAT Asn 610	TTC Phe	TCA Ser	TAT Tyr	CAG Gln	TCT Ser 615	TAT Tyr	CAC His	GAT Asp	GAG Glu	TCC Ser 620	ATG Met	3912
TTG Leu	TCC Ser	GCA Ala	CAC His 625	AAT Asn	TTG Leu	AAT Asn	AGT Ser	GCC Ala 630	GAC Asp	TTG Leu	ATC Ile	TAT Tyr	AAA Lys 635	TCT Ser	TTG Leu	3960
AGT Ser	CAC His	TCT Ser 640	GGA Gly	CTA Leu	GAT Asp	GAT Asp	GGC Gly 645	TTG Leu	GAA Glu	CAG Gln	GGC Gly	TTG Leu 650	AAT Asn	CGT Arg	TCT Ser	4008
TTA Leu 655	AGC Gly	GGA Leu	CTG Asp	TTA Leu	CAA Gln 660	AAC Asn	CAA Gln	AAC Asn	AAG Lys	AAG Lys 665	AAT Asn	CTA Leu	TGG Trp			4053

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TAATATATAC	TTCCATTATT	CTATGATTAT	AGAGTTTGTT	TGGTATTTGT	ATATCGCACG	4113
ATACAAGTAA	TGAGGGGTGC	TTACACAAGA	TAAAAGATAA	AAAAATATAT	ATATATAATA	4173
AAAACCATCA	AAAACACCAT	TGAAAAAAAA	TATAAAAAAA	AAAAAAATA	ACCGAATATG	4233
AATATGAAAT	TAATGATCAT	GATGAAGTTA	ATTTTTACTG	AGAAACGTCA	CCTAATGTCG	4293
ATGAAACGAT	GATAATGAAT	GAATGATGAG	GCTACTTTAA	GTAACGCAAT	GTAATCAAGC	4353
CAAAATTATC	CCTCTTTTTT	TTTTTCCCT	CTTTTGAGAT	TTTATTTTTA	ACCTACTACT	4413
TACTTTTTTT	TTTTGAACGT	TCTTTTCCCA	CATACTTTTA	TATATGGTAT	TTATATGTAC	4473
GATGTTTAAT	CACAGAGATG	TTTCTACCTT	ACTCGATATT	GTTTTTGCAT	TAATTGATAA	4533
CTTGCTCACT	GCATCATTGG	CGGTATTTGT	AGTATATAGA	AAGTCGGGTA	ACAATAATTT	4593
ATTGACATTT	CTTTGTTTAC	AATGATCAGA	GAAGAGCAGA	AAGTTTCATA	GTCAAACGTT	4653
CAGGCCAATT	GAACAAGAAA	TTATTGTTTT	TTTAGTCGT	TGAGTGTTCA	ACTGACATGC	4713
TATTTTGGTG	GTTCTTGATT	AATTGGGGGC	TTCATTGTTT	GAAATAAAGA	GTCTGGGAAA	4773
TAGCACAGAA	ACAAAGCATA	TTAAAAGAGG	CAAAAGAAGA	AAGAACGAAT	ATAAAAGGTA	4833
AAAAAGGAAA	AGCATTGCTA	TTCTTTTCTC	ATAGGTGTTA	TTCATACCGC	CCTCTCTCTT	4893
CTTCCTTCTT	CATTAATTAG	TCTCCGTATA	ATTTGCAGAT	AATGTCATTA	ACAGCAAACG	4953
ACGAATCGCC	AAAACCCAAA	AAAAATGCAT	TATTGAAAAA	CTTAGAGATC	GATGATCTGA	5013
TACATTCTCA	ATTTGTCAGA	AGCGATACAA	ATGGACATAG	AACTACAAGA	CGACTATTCA	5073
ACTCCGATGC	CAGTATATCA	CATCGAATAA	GAGGAAGTGT	TCGGTCTGAT	AAAGGCCTTA	5133
ATAAAATAAA	AAAAGGGTTG	ATTTCCAGC	AGTCCAAACT	TGCGTCAGAA	AATTCTTCTC	5193
AAAATATCGT	TAATAGGGAC	AATAAGATGG	GAGCAGTAAG	TTCCCCATT	ATTGAACCTA	5253
ATATTGAAGT	CAGCGAGGAG	TTGAAGGTTA	GAATTAAGTA	TGATTCTATC	AAATTTTTCA	5313
ATTTTGAAAAG	ACTAATATCT	AAATCTTCAG	TCATAGCACC	TTTAGTTAAC	AAAAATATAA	5373
CATCATCCGG	TCCTCTAATC	GGGTTTCAAA	GAAGAGTTAA	CAGGTAAAG	CAAACATGGG	5433
ATCTAGCAAC	CGAAAACATG	GAGTACCCAT	ATTCTTCTGA	TAATACGCCA	TTCAGGGATA	5493
ACGATTCTTG	GCAATGGTAC	GTACCATACG	GCGGAACAAT	AAAAAAAATG	AAAGATTTCA	5553
GTACAAAAAG	AACTTTACCC	ACCTGGGAAG	ATAAAATAAA	GTTTCTTACA	TTTTTAGAAA	5613
ACTCTAAGTC	TGCAACGTAC	ATTAATGGTA	ACGTATCACT	TTGCAATCAT	AATGAAACCG	5673
ATCAAGAAAA	CGAAGATAGG	AAAAAAGGA	AAGGGAAAGT	ACCAAGAATC	AAAAATAAAG	5733
TGTGGTTTTTC	CCAGATAGAA	TACATTGTTC	TTCGAAATTA	TGAAATTAAA	CCTTGGTATA	5793
CATCTCCTTT	TCCGGAACAC	ATCAACCAAA	ATAAAATGGT	TTTTATATGT	GAGTTCTGCC	5853
TAAAATATAT	GACTTCTCGA	TATACTTTTT	ATAGACACCA	ACTAAAGTGT	CTAACTTTTA	5913
AGCCCCCGG	AAATGAAATT	TATCGCGACG	GTAAGCTGTC	TGTTTGGGAA	ATTGATGGGC	5973

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GGGAGAATGT CTTGTATTGT CAAAATCTTT GCCTGTTGGC AAAATGTTTT ATCAATTCTA      6033
AGACTTTGTA TTACGATGTT GAACCGTTTA TATTCTATAT TCTAACGGAG AGAGAGGATA      6093
CAGAGAACCA TCCCTATCAA AACGCAGCCA AATTCCATTT CGTAGGCTAT TTCTCCAAGG      6153
AAAAATTCAA CTCCAATGAC TATAACCTAA GTTGTATTTT AACTCTACCC ATATACCAGA      6213
GGAAAGGATA TGGTCAGTTT TTGATGGAAT TTTCATATTT ATTATCCAGA AAGGAGTCAA      6273
AATTTGGAAC TCCTGAAAAA CCATTGTCGG ATTTAGGATT ATTGACTTAC AGAACGTTTT      6333
GGAAGATAAA ATGTGCTGAA GTGCTATTAA AATTAAGAGA CAGTGCTAGA CGTCGATCAA      6393
ATAATAAAAA TGAAGATACT TTTCAGCAGG TTAGCCTAAA CGATATCGCT AAACCTAACAG      6453
GAATGATACC AACAGACGTT GTGTTTGGAT TGGAACAAC TCAAGTTTTG TATCGCCATA      6513
AAACACGCTC ATTATCCAGT TTGGATGATT TCAACTATAT TATTAAAATC GATTCTTGGA      6573
ACAGGATTGA AAATATTTAC AAAACTTGGA GCTCAAAAAA CTATCCTCGC GTCAAATATG      6633
ACAAACTATT GTGGGAACCT ATTATATTAG GGCCGTCATT TGGTATAAAT GGGATGATGA      6693
ACTTAGAACC CACCGCATTA GCGGACGAAG CTCTTACAAA TGAAACTATG GCTCCGGTAA      6753
TTTCGAATAA CACACATATA GAAAACATA ACAACAGTAG AGCACATAAT AAACGCAGAA      6813
GAAGAAGAAG AAGAAGTAGT GAGCACAAAA CATCCAAGCT T                               6854

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(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2814 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..696

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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GAA TTC CAA TAC ACC AAA CAG CTG CAT TTC CCT GTG GGG CCC AAA TCC      48
Glu Phe Gln Tyr Thr Lys Gln Leu His Phe Pro Val Gly Pro Lys Ser
 1             5             10             15

ACA AAC TGT GAG GTA GCG GAA ATT CTT TTA CAC TGC GAC TGG GAA AGG      96
Thr Asn Cys Glu Val Ala Glu Ile Leu Leu His Cys Asp Trp Glu Arg
             20             25             30

TAC ATA AAT GTT TTA AGT ATA ACA AGA ACA CCA AAT GTT CCT AGT GGT      144
Tyr Ile Asn Val Leu Ser Ile Thr Arg Thr Pro Asn Val Pro Ser Gly
             35             40             45

ACC AGT TTC AGC ACC AGA ACG AGG TAC ATG TTC CGA TGG GAT GAC CAG      192
Thr Ser Phe Ser Thr Arg Thr Arg Tyr Met Phe Arg Trp Asp Asp Gln
 50             55             60

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GGG Gly 65	CAA Gln	GGT Gly	TGC Cys	ATA Ile	TTA Leu 70	AAA Lys	ATA Ile	AGT Ser	TTT Phe	TGG Trp 75	GTG Val	GAC Asp	TGG Trp	AAC Asn	GCA Ala 80	240	
TCC Ser	AGT Ser	TGG Trp	ATC Ile	AAG Lys 85	CCA Pro	ATG Met	GTA Val	GAG Glu	AGC Ser 90	AAT Asn	TGT Cys	AAA Lys	AAT Asn	GGA Gly 95	CAA Gln	288	
ATT Ile	AGC Ser	GCC Ala	ACT Thr 100	AAG Lys	GAC Asp	TTG Leu	GTA Val	AAG Lys 105	TTA Leu	GTC Val	GAA Glu	GAA Glu	TTT Phe 110	GTA Val	GAG Glu	336	
AAA Lys	TAC Tyr	GTG Val 115	GAA Glu	TTG Leu	AGC Ser	AAA Lys	GAA Glu 120	AAA Lys	GCA Ala	GAT Asp	ACA Thr	CTC Leu 125	AAG Lys	CCG Pro	TTG Leu	384	
CCC Pro	AGT Ser 130	GTT Val	ACA Thr	TCT Ser	TTT Phe	GGA Gly 135	TCA Ser	CCT Pro	AGG Arg	AAA Lys	GTG Val 140	GCA Ala	GCA Ala	CCG Pro	GAG Glu	432	
CTG Leu 145	TCG Ser	ATG Met	GTA Val	CAG Gln	CCG Pro 150	GAG Glu	TCG Ser	AAA Lys	CCA Pro	GAA Glu 155	GCT Ala	GAG Glu	GCG Ala	GAA Glu	ATC Ile 160	480	
TCA Ser	GAA Glu	ATA Ile	GGC Gly 165	AGC Ser	GAC Asp	AGA Arg	TGG Trp	AGG Arg	TTT Phe 170	AAC Asn	TGG Trp	GTG Val	AAC Asn	ATA Ile 175	ATA Ile	528	
ATC Ile	TTG Leu	GTG Val	CTC Leu 180	TTG Leu	GTG Val	TTA Leu	AAT Asn	CTG Leu 185	CTG Leu	TAT Tyr	TTA Leu	ATG Met	AAG Lys 190	TTG Leu	AAC Asn	576	
AAG Lys	AAG Lys	ATG Met 195	GAT Asp	AAG Lys	CTG Leu	ACG Thr	AAC Asn 200	CTC Leu	ATG Met	ACC Thr	CAC His	AAG Lys 205	GAC Asp	GAA Glu	GTT Val	624	
GTA Val 210	GCG Ala	CAC His	GCG Ala	ACT Thr	CTA Leu	TTG Leu 215	GAC Asp	ATA Ile	CCA Pro	GCC Ala	CAA Gln 220	GTA Val	CAA Gln	TGG Trp	TCA Ser	672	
AGA Arg 225	CCA Pro	AGA Arg	AGG Arg	GGA Gly	GAC Asp 230	GTG Val	TTG Leu	TAACAGAGTA			ATCATGTAAT		ATTGTATGTA			726	
AGGTTATGTA					TGTTTCGTATG		GTATGGAAAA		AAAAAAAAAA			AAAGGATGCT		ATGTGGAGAA			786
TGTAAGGCGT					GGTAGCTCCG		GATAATTCAG		TCTGTAGGCT			TCATCACGGG		CAGTGGCCTG			846
ACTCTGAGAG					CTTGCTCCGG		TATTAAGTTG		TGCGTTTGAA			ATTTTCTGGA		AAAAAGAAAT			906
TGATTGGTTG					AAGCTATACT		CGTCGAAAGA		TTTCTTCGGC			AGTGGTTGTT		GCTCCACCTG			966
CACGGGAGTT					GTGTTTGCGT		TTATGTTTCGG		CTTGGCTATA			TTATTAGCGA		GTGATGTTTG			1026
CAATTTGCTG					TATTGAGAAT		CAATTTGGGT		GCGTAAGCTT			TCAATAATTT		TGCAGACCGC			1086
AGGCACTTCC					AACTTTATGA		GTTGCAGGTA		TTCTCTTTTA			TGAATATACG		ATGACGACGA			1146
TGACGACGAC					GCATCCATGC		GCAAAAGCTC		AGGGTGTCTA			GATAGTTTGT		TAGTCAATAA			1206
ATCCACATAT					CTAAAATAAT		AAATAAACGA		CAGCGACAAG			TCGTTGGCCT		GGAACGCACA			1266

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ATTGAAGCGA	TAATCCTTAG	CGTCCTGAAC	CAATTGCTTA	CTAGACTTCA	TGACCTACCG	1386
GGGCCAGATA	AAGATGCGGA	AGGAAGAGAA	AAAATGTATA	GTGGTTGGTG	AACCGCAACA	1446
ATAATTCGTG	CCAACACTTT	AATCGAAGCA	AAAATTGTCT	TGTATGTTAT	TAATATTATC	1506
TATCTAACCA	TTGATTTACG	TATAAAACTG	TCGATGCTCA	TCGCCTAGCA	ATGAAAAAAT	1566
TTTTTCTTTT	TTTTTTCATT	ATTTCTCTTT	GTTGCGTACT	TTTTTTCATT	GCGTTTCGCG	1626
GCAAAAGCGA	TTCGAGTTGA	CTGGAAGTGT	GTTATACTAT	AAAAAGTGTA	TATGCCTATT	1686
TTTGGTTCTG	ATCTTTACTT	TACTGTTAAG	TACTGGCTGA	GGCAGTAGAC	TCTGCCTCTG	1746
TTACGGCAGC	GGTATTCGCC	TCGGCATCAG	CAGCCGCCCA	CGGTAGAGTA	GGTTCGTGTG	1806
TTTTGACGTT	TGCCAAGGTA	CTGTCCAAAT	GCTCCTTCAG	CAAGGCCTCA	TTACTTTCCT	1866
TCTCCGGACC	CACCGATTGC	GTGATCTCCT	GTACACGGTT	CAAGAACTTG	TTCAAATTGT	1926
AGCCCGCAGC	AGCATCAGAG	ACTTCTTGTG	TGTAAGGGAC	ACCCCTCAAC	TCCTTGACTC	1986
TTCTTTTGTG	CACTTTGCCC	TTTAAATGCG	TTTTTAAACGC	TATAGCAGTC	TCCATGTATT	2046
TGGCACAGTG	TATGCAATAG	TGCTGACCAA	GGCCCGGTTT	GGTTTCATCC	AATGGCTGGT	2106
TCAGAAGCTT	CTGTACTGAT	TCCTTGGTGG	ACAAATCGTT	ATAGATCAGG	TCCAAGTCTC	2166
GTGTTCTTCT	TTTAGTCTTG	TATCTCTTCA	CCGAATATCT	ACCCATGATG	CGCTATTGTT	2226
TTATCTTCAC	TTGTCTGTGT	GTTTAACTGC	CTTTCAATTC	ACCTCATCTC	ATCTCCCGCT	2286
ACTTTCCATA	TATAAAAGCA	AAATTAATTT	GCTTTTTCCC	CTGTCAGTAT	AAAAAAATTT	2346
TCCGCAGGAT	ATAGAAAAAA	AAGAAATGAA	ATTATAGTAG	CGGTTATTTT	CGTGGGGTGC	2406
TTTTTTACAC	CTGTACATCT	TTTCCCTCCG	TACATTTTTT	TTATTTTTTT	TTTGGGGTTT	2466
TTTTTTTCGA	TATTTTTTCCC	TCCGAAACTA	GTTAGCACAA	TAATGCTGAC	TAAGGAAACT	2526
TTTCATCTCA	GAATTGATGG	TCAGTTTGGT	TTCTCTAGAG	AATAGTTTAT	AAAAAGATGT	2586
TGATGTGGAG	CAACCATTTA	TACATCCTTT	CCGCAAGTGC	TTTTGGAGTG	GGACTTTCAA	2646
ACTTTAAAGT	ACAGTATATC	AAATAACTAA	TTCAAGATGG	CTAGAAGACC	AGCTAGATGT	2706
TACAGATACC	AAAAGAACAA	GCCTTACCCA	AAGTCTAGAT	ACAACAGAGC	TGTTCCAGAC	2766
TCCAAGATCA	GAATCTACGA	TTTGGGTAAG	AAGAAGGCTA	CCGTCGAT		2814

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
 1 5 10

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WHAT IS CLAIMED IS:

1. A host cell transformed or transfected with DNA comprising:

a repressor gene encoding a repressor protein, said repressor gene under transcriptional control of a promoter;

a selectable marker gene encoding a selectable marker protein; said selectable marker gene under transcriptional control of an operator; said operator regulated by interaction with said repressor protein;

a first recombinant fusion protein gene encoding a first binding protein or binding fragment thereof in frame with either a DNA binding domain of a transcriptional activating protein or a transactivating domain of a transcriptional activating protein; and

a second recombinant fusion protein gene encoding a second binding protein or binding fragment thereof in frame with either a DNA binding domain of a transcriptional activating protein or a transactivating domain of a transcriptional activating protein, whichever domain is not encoded by the first fusion protein gene, said second binding protein or binding fragment thereof capable of interacting with said first binding protein or binding fragment thereof such that interaction of said second binding protein or binding fragment thereof and said first binding protein or binding fragment thereof brings into proximity a DNA binding domain and a transactivating domain forming a functional transcriptional activating protein; said functional transcriptional activating protein acting on said promoter to increase expression of said repressor gene.

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2. The host cell of claims 1 wherein said DNA binding domain and said transactivating domain are derived from a common transcriptional activating protein.

3. The host cell of claim 1 wherein one or more of the repressor gene, the selectable marker gene, the first recombinant fusion protein gene, and the second recombinant fusion protein gene are encoded on distinct DNA expression constructs.

4. The host cell of claim 1 wherein said selectable marker protein is an enzyme in a pathway for synthesis of a nutritional requirement for said host cell such that expression of said selectable marker protein is required for growth of said host cell on media lacking said nutritional requirement.

5. The host cell of claim 1 wherein said host cell is a yeast cell or a mammalian.

6. The host cell of claim 2 wherein said selectable marker gene encodes HIS3;

7. The host cell of claim 2 wherein said repressor protein gene encodes a tetracycline resistance protein;

8. The host cell of claim 2 wherein said operator is a *tet* operator.

9. The host cell of claim 2 wherein said promoter is selected from the group consisting of the LexA promoter, the alcohol dehydrogenase promoter, the Gal4 promoter.

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10. The host cell of claim 2 wherein said DNA binding domain derived from a protein selected from the group consisting of LexA and Gal4.

11. The host cells of claim 2 wherein said transactivating domain is derived from a protein selected from the group consisting of VP16 and Gal4.

12. The host cell of claim 2 wherein the first binding protein is CREB and the second binding protein is CBP.

13. The host cell of claim 2 wherein the first binding protein is Tax and the second binding protein is SRF.

14. The host cell of claim 2 wherein the first binding protein is casein kinase I and the second binding protein is CREB.

15. The host cell of claim 2 wherein the first binding protein is AKAP 79 and the second binding protein is selected from the group consisting of RI, RII and calcineurin.

16. A method to identify an inhibitor of binding between a first binding protein or binding fragment thereof and a second binding protein or binding fragment thereof comprising the steps of:

- a) growing host cells of any one of claims 1 through 15 in the absence of a test compound and under conditions which permit expression of said first binding protein or binding fragment thereof and said second binding protein or binding fragment thereof such that said first binding protein or fragment thereof and second binding protein or binding fragment thereof interact bringing

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into proximity said DNA binding domain and said transactivating domain forming said functional transcriptional activating protein; said transcriptional activating protein acting on said promoter to increase expression of said repressor protein; said repressor protein interacting with said operator such that said selectable marker protein is not expressed;

- b) confirming lack of expression of said selectable marker protein in said host cell;
- c) growing said host cells in the presence of a test compound; and
- d) comparing expression of said selectable marker protein in the presence and absence of said test compound wherein increased expression of said selectable marker protein is indicative that the test compound is an inhibitor of binding between said first binding protein or binding fragment thereof and said second binding protein or binding fragment thereof.

17. The method of claim 16 wherein

the host cell is a yeast cell;
the selectable marker gene encodes HIS3;
transcription of the selectable marker gene is regulated by the *tet* operator;
the repressor protein gene encodes the tetracycline resistance protein;
transcription of the tetracycline resistance protein is regulated by the LexA promoter;
the DNA binding domain is derived from LexA; and
the transactivating domain is derived from VP16.

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18. The method of claim 16 wherein
 - the host cell is a yeast cell;
 - the selectable marker gene encodes HIS3;
 - transcription of the selectable marker gene is regulated by the *tet* operator;
 - the repressor protein gene encodes the tetracycline resistance protein;
 - transcription of the tetracycline resistance protein is regulated by the alcohol dehydrogenase promoter;
 - the DNA binding domain is derived from LexA; and
 - the transactivating domain is derived from VP16.
19. A kit to identify an inhibitor of binding between a first binding protein or binding fragment thereof and a second binding protein or binding fragment thereof, said inhibitor identified by the method of claim 16.

1/1

FIGURE 1

